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UNIVERSITÄT ZU SENDAI
AND
MITTEILUNGEN ÜBER ALLGEMEINE PATHOLOGIE
UND PATHOLOGISCHE ANATOMIE

OHIO STATE UNIVERSITY
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Vol. 69, Nos. 2–3, February 25, 1959

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THE TOHOKU UNIVERSITY

SENDAI, JAPAN

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The Tohoku Journal of Experimental Medicine, Vol. 69, Nos. 2-3, 1959
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The Relation of the Electric Threshold and Accommodation in the Human Retina to Brightness Discrimination

By

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(Received for publication, September 13, 1958)

Introduction

The author investigated in a previous paper[1] the relation between the electric threshold (V) and the radiance (I) of the adapting colored light, and reported that there were two different types in the form of the V-log I curve depending upon the different time-constant (RC) of the exponentially increasing current, i.e., one was a group of the curves for RC shorter than 25 msec., and the other was a group of those for RC longer than 75 msec. For mathematical expression of these V-log I curves, he had assumed the following equation for the relation between the electric threshold (V) and the intensity (I) of the adapting light,

$$V = A \log \Delta I = A \left[\log \left(\Delta I / I\right) + \log I\right] \quad \text{(1)},$$

where A is a proportionality constant and ΔI is the least perceptible brightness increment to be added to the brightness (I) of the background, so that $\Delta I / I$ means the Weber fraction (W). Using the data of Steinhardt and Blackwell's experiments[2][3] concerning the Weber fraction, we obtained V by equation (1). The thus calculated V-log I curve was well consistent in shape with the curves obtained experimentally by the currents of RC longer than 75 msec., however it did not agree with those obtained by the currents of RC shorter than 25 msec.

In this paper, the author attempted to get a mathematical formula applicable not only to the former case but also to the latter case. And, in order to confirm the validity of such an hypothetical formula, the relation between the accommodation constant (Hill's λ[4]) and the intensity of the adapting light was investigated precisely with the eyes adapted to monochromatic lights of varying wavelengths.

Experimental

Methods

As a stimulating electrode, a silver plate of 2×3 cm. in size was inserted between the upper lip and gum, while another large silver plate was placed under the right sole, being immersed into 1% saline as an indifferent electrode. Taking the electrical phosphene as an index of excitation, the electric thresholds (V) were determined for the varying time-constants (RC) of the stimulating current. Measurements of V were made by the same procedure as reported in the previous paper[5]. Every adapting colored light of different wavelengths was isolated by a set of metal interference filters of Lange & Co., whose spectral transmittance must be referred to Yaegashi's paper[6]. The radiance (I) of the surface emitting the adapting light was measured by a system of a thermopile and a galvanometer.

Results

a) Dependence of the form of the accommodation curve on the wavelength and on the radiance of the adapting light

In order to look over the dependence of the accommodation curve on the wavelength of the adapting light, three monochromatic rays of 430, 550 and 700 mμ in wavelength were first employed as the adapting light. In these cases, these monochromatic lights used were of an equal energy level high enough for photopic vision. The curves obtained are shown in Fig. 1 (A).

As can be seen in Fig. 1 (A), the slope of the curve becomes steeper in the order of 700, 430 and 550 mμ. This fact suggests that the change in slope of the curves is due to the spectral luminosity of the adapting lights. The curves for 430 and 700 mμ show an abrupt inflexion at about 50-80 msec. in RC, while the curve for 550 mμ does not show any inflexion at this range of RC. All these findings are well in conformity with the results which were obtained by Sato, Suzuki and Akihama[5] with the eye adapted to various intensity levels of white light. It was found that the form of the accommodation curve depends on the intensity of the adapting light, and that an intensity level to produce a straight form of the accommodation curve without inflexion, is approximately 1.2×10 lux. Accordingly, the radiance of the adapting light of 550 mμ used in this experiment would give the same illuminance as 1.2×10 lux of white light.

Secondly, the dependence of the form of the accommodation curve on the radiance of the adapting light was investigated for varying wavelengths at 430, 500, 550, 670 and 700 mμ, and some data of the measurements were plotted in Fig. 1 (B), (C) and (D), others were shown in Table I.

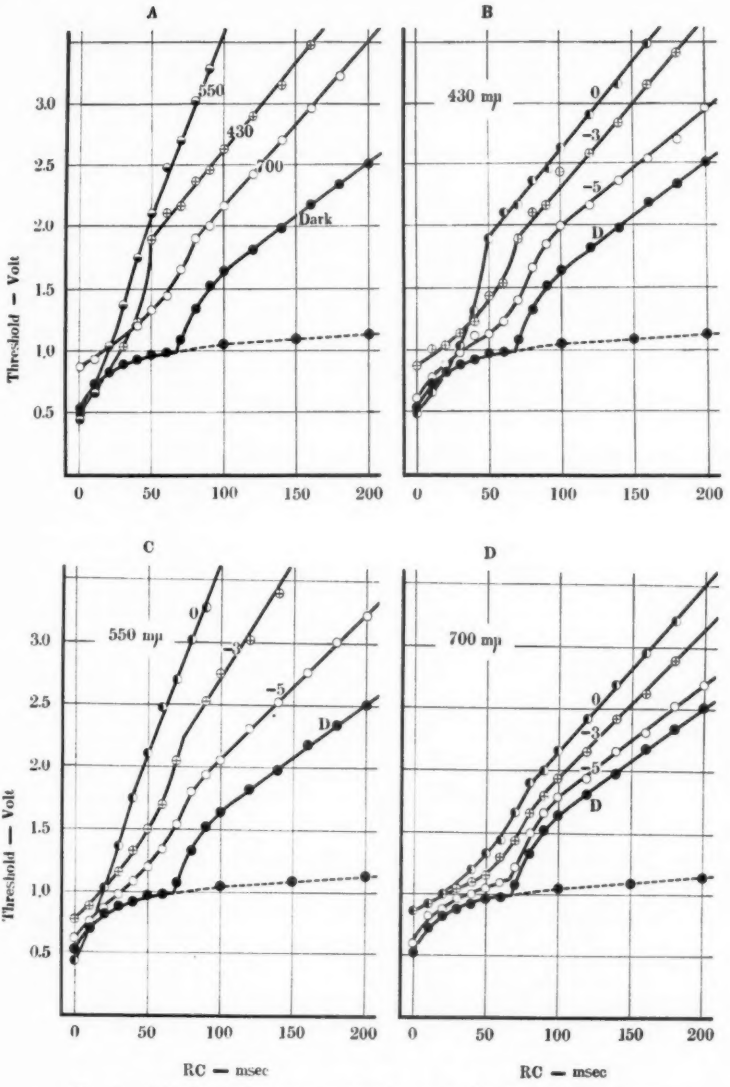

Fig. 1. (A) shows the accommodation curves for three different wavelengths of the adapting light whose radiances were fixed at an equal energy enough for photopic vision. Numbers on the curves are wavelength in mμ. (B), (C) and (D) show the dependence of the accommodation curve on the radiance of the adapting light of 430, 550 and 700 mμ in wavelength respectively. Filled circles are data obtained from the dark adapted eyes.

TABLE I

Dependence of the Electric Threshold on the Time-constant and on the Radiance of the Adapting Light

Electric thresholds are shown in volt. Subject: M. Sato (Exp. No. 560120).

Radiance→ / Time const. in msec. ↓	(500 mμ)				(670 mμ)		
	Dark	10^{-5}	10^{-3}	1	10^{-5}	10^{-3}	1
0	0.52	0.80	0.75	0.54	0.62	0.88	0.78
10	0.68	0.95	0.82	0.82	0.80	0.95	0.85
20	0.80	1.03	0.90	1.08	0.85	1.05	1.03
30	0.88	1.11	1.00	1.33	0.95	1.10	1.10
40	0.98	1.22	1.13	1.58	0.99	1.19	1.26
50	0.96	1.38	1.38	1.85	1.05	1.30	1.44
60	0.98	1.58	1.79	2.10	1.10	1.37	1.91
70	1.06	1.80	2.05	2.36	1.30	1.54	1.92
80	1.32	2.10	2.26	2.63	1.52	1.74	2.00
90	1.50	2.26	2.42	2.90	1.66	1.90	2.26
100	1.64	2.42	2.54	3.15	1.80	2.00	2.36
120	1.82	2.63	2.90	3.60	1.99	2.26	2.63
140	1.98	2.96	3.20		2.16	2.53	2.96
160	2.16	3.15	3.59		2.36	2.76	3.28
180	2.34	3.42			2.48	3.02	3.47
200	2.52				2.70	3.28	

As can be seen in Fig. 1 (B), (C) and (D), the slope of the curves becomes steeper as the radiance is raised progressively. The rate of variation in slope with increase in radiance of the adapting light is also dependent upon the wavelength, that is to say: this rate increases in the order of 700, 430 and 550 mμ.

b) Relation between Hill's λ and log-radiance (I) of the adapting light

As shown in Fig. 1, most accommodation curves show an inflexion at about 50–80 msec. in RC. The curve is divided by this inflexion into a "left section" and a "right section" for time-constants shorter and longer than this critical value of RC, respectively. Now, from both sections of an accommodation curve, two values of Hill's λ were obtained separately. The data obtained from the "left section" were plotted in Fig. 2 (A) and those obtained from the "right section" in Fig. 2 (B).

The value of λ in Fig. 2 (A) decreases rapidly showing an upward concave curve with increase in radiance from dark to 10^{-3} unit (relative value). Then, it decreases again, marking a faint inflexion when the radi-

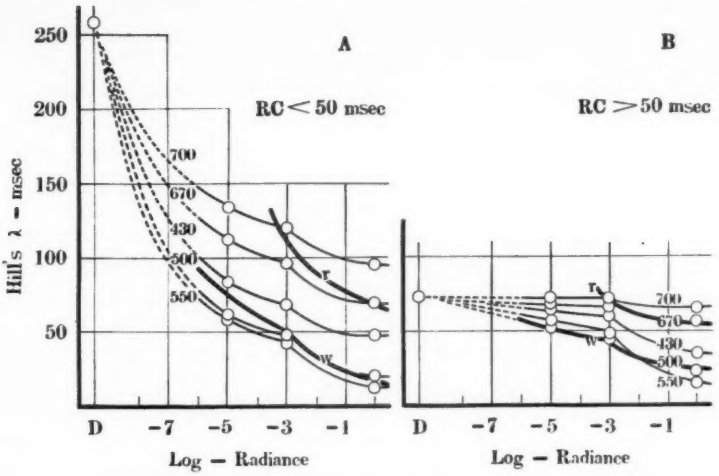

Fig. 2. The relation between Hill's λ and log-radiance of the adapting light. (A) and (B) are cases for RC<50 msec. and RC>50 msec. respectively. Numbers on the curves are wavelength of the adapting light in mμ. D means complete dark. The thick curves denoted by r and w show the λ-log I relation calculated from the equation (5′) by using Steinhardt's data for the red and white light respectively.

ance is raised further. On the other hand, the value of λ in Fig. 2 (B) decreases gradually with a gentle slope till the radiance arrives at 10^{-3} unit, and then decreases rapidly, showing a clear break with further increase in radiance. Thus, there was found a remarkable difference in form between both kinds of the λ-log I curves obtained from the left and right sections. This result seems to indicate a different seat of excitation in the retina, depending on whether the stimulating current of RC is longer or shorter than 50 msec., as inferred by Mita *et al.*[7].

5 curves of λ-log I relation in Fig. 2 (A) or (B) show the systematic vertical displacement according to the wavelength of the adapting light. As can be seen in Fig. 2 (B), the magnitudes of these vertical displacements are proportional to the spectral luminosity of the adapting monochromatic lights respectively. Attention should be directed to the fact that the order of this vertical shifting differs depending upon whether the intensity range of the adapting light is scotopic or photopic. That is, within the scotopic range, the curves for varying wavelengths shift downwards in a parallel relation to the scotopic luminosity of the spectrum, while in the photopic range, the curves to the photopic luminosity of the spectrum. It is further to be noted that the hue of the adapting light can be perceived at first at

the radiance corresponding to this break on the curve. The curves for the red end of the spectrum show little variation within a scotopic range of the radiance. This is obviously due to the low stimulating efficiency of red light for rod. From these facts, it can be deduced that the inflexion on the λ-log I curves at about 10^{-3} unit in radiance is caused by the independent function of the two types of receptors, namely rods and cones, and it was assumed that the neurons stimulated by the currents of RC longer than 50 msec. may be closely related to the primary receptive neurons. On the other hand, in Fig. 2 (A), the order of the downward shifting of the curves corresponds always to the photopic luminosity of the spectrum, irrespective of whether the adaptaion was scotopic or photopic. Moreover, the curves for the red end of the spectrum show a far steeper slope than in the former case (Fig. 2 (B)) even within a scotopic range in radiance. In addition, the inflexion of the λ-log I curve at about 10^{-3} unit does not appear so clearly as in the case of Fig. 2 (B). From these facts, it seems likely that the neural elements stimulated by the current of RC shorter than 50 msec. would not be so closely related to the receptor neurons as in the former case, but probably to some other secondary neurons, such as the bipolar cells and horizontal cells.

Discussion

a) Dependence of the electric threshold (V) on the radiance (I) of the adapting colored light and on the time-constant (RC) of the stimulating current

As the author reported in the previous paper[1], equation (1) was applicable only to the case of RC longer than 75 msec., but it was not applicable to the case of RC shorter than 25 msec. In order to get a mathematical formula applicable not only to the former case but also to the latter, the author introduced a parameter p into equation (1), as follows:

$$V=A\ (\log W^{p}+\log I)+B \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (2)$$

where B is a constant independent of I and W. Equation (1) can be regarded as the special case for $p=1$ in equation (2). Now, using Steinhardt's data[2] for I and W, and putting $p=1$, 1.5, 2, 3, 3.5, we get five curves from equation (2) as shown in Fig. 3, where $A=3.1^{-1}$, $B=3.7-0.71p$ in the cases of $p\leqq 2$ and $B=3.7-0.55p$ in the cases of $p\geqq 3$. As can be seen in Fig. 3, each curve for $p=1$, 1.5, 2, 3, 3.5 agrees approximately with the experimental data (shown by circles) for time-constant (RC)=100, 75, 50, 25, 0 msec. respectively. Furthermore, if we compare the above calculated curve with the V-log I curves which were reported by Mita, Fujimaki & Takahashi[7] and Mita, Fujimaki & Yaegashi[8], in the case of stimulation with the sinusoidal alternating current, it is found that the

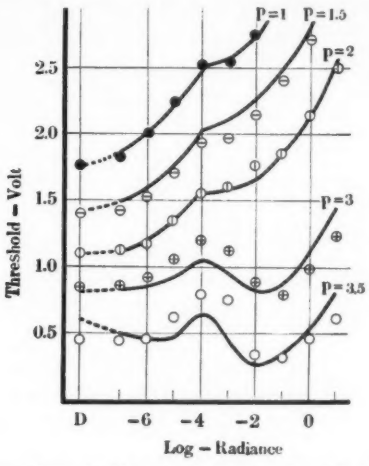

Fig. 3. Curves show the V-log I relation calculated from equation (2) by using the Steinhardt's data and by putting p=1, 1.5, 2, 3, 3.5. Circles show the experimental data obtained with the eye adapted to monochromatic light of 550 mμ in wavelength. The time-constants of the stimulating currents used are as follows, ●: 100 msec., ⊖: 75 msec., ⓘ: 50 msec., ⊕: 25 msec., ○: 0 msec.

V-log I curves for p=1, 3, 5 in equation (2) are quite similar to those for frequencies at 3, 30 and 1000 cps. respectively. These facts demonstrate that the parameter p in equation (2) can be regarded as a function of the experimental condition of electrostimulation, such as time-constant (RC) and frequency of the stimulating currents. It is further to be noted that in the cases of electrostimulation of the eye with A. C. also, V-log I curves can be represented satisfactorily in a single equation (2) by taking some suitable value of p.

Equation (2) can be expressed also in another form,

$$V=A \log \Delta I \ (\Delta I/I)^{p-1}+B \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (3)$$

In the case for p=1, equation (3) means that the electric threshold is simply proportional to the logarithm of the least perceptible brightness increment (ΔI), just as if the structures stimulated were the primary receptive neurons. On the other hand, in the cases for p=2 or more, the equation (3) expresses that the electric threshold is not simply proportional to the log I, so that it should be determined by another more complicated mechanism of excitation than in the case for p=1. These facts also afford evidence supporting the Mita *et al.*'s working hypothesis with regard to the different seat of stimulation in the retina.

b) Dependence of the Hill's λ upon the intensity of the adapting light

The fact that Hill's accommodation constant λ of the human retina for electrostimulation varies with varying intensities of the adapting light, was first reported by Motokawa & Iwama[9]. Now, a question arises why λ varies depending on the adaptation levels, and what mechanism causes this phenomenon. In this connection, the author attempted to answer the problem from the viewpoint of the brightness discrimination, as stated above. As the parameter p is considered to be a function of the time-constant (RC) of the stimulating current, we get the following formula, by differentiating the equation (2) with respect to RC,

$$\partial V/\partial t = A \ (\log W) \ \partial p/\partial t \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (4),$$

where t denotes the RC. According to Hill's theory, the accommodation constant λ is presented by the reciprocal of the tangent of the V-RC curve when λ/k is large and RC of the stimulating current is far larger than k and λ. Then, we obtain,

$$\partial V/\partial t = A(\log W) \ \partial p/\partial t = V_0/\lambda \quad \ldots\ldots\ldots\ldots\ldots \quad (5),$$

or,
$$V_0/\lambda = A(\log \Delta I/I) \ \partial p/\partial t \quad \ldots\ldots\ldots\ldots\ldots \quad (5'),$$

where V_0 means the rheobase. V_0, λ and A in the equation (5) were already known by this experiment and log W is referable to Steinhardt's data[2]. Therefore, two values of $\partial p/\partial t$ can be calculated by the equation (5), i.e., -0.027 and -0.042 for both cases of RC shorter and longer than 50 msec. respectively. Substituting these two values of constants for the equation (5′), the λ-log I curves obtained are shown by thick curves in Fig. 2 (A) and (B), respectively. The r curves were obtained by using Steinhardt's data[2] for red light (test field of 20°), and the w curves by using his data for white light (test field of 20°). As can be seen in Fig. 2 (A) and (B), the r and w curves are approximately corresponding to the respective thin curves for red and green, which were obtained actually in this experiment, although the r and w curves obtained from the Steinhardt's data covered only a narrow range of radiance. Thus, this consistent results of the experimental data with the formula (5′), indicate that $\partial p/\partial t$ can be expressed approximately by two constants corresponding to the time-constants shorter or longer than 50 msec., as mentioned above. Integrating the equation $\partial p/\partial t = \text{constant}$, we obtain

$$p = -0.027\,t + C_1 \qquad \text{for } t < 50 \text{ msec.},$$
$$p = -0.042\,t + C_2 \qquad \text{for } t > 50 \text{ msec.},$$

respectively, where C_1 and C_2 are constants of integration. However, when we put

$p=9.3\ e^{-t/285}-5.8$ for $t<50$ msec.,
$p=-0.042\ t+5.2$ for $t>50$ msec.,

respectively, far better agreement of these formulae with the actual data of the p–t relation can be obtained.

As discussed above, it is noteworthy that the correlation among the electric threshold (V), brightness discrimination and the time-constant (RC) of the stimulating current was elucidated by expressing in an experimental formula, and that the different values of parameter p in the formula relate closely to the site of electrostimulation in the human retina. Another deduction worth noting is that the accommodation constant of the retina is inversely proportional to the Weber fraction in brightness discrimination.

Summary

Using, as an index of excitation, the electrical phosphene elicited by the exponentially increasing current running through the eyeballs, the relation of the electric threshold and accommodation in the human retina to brightness discrimination was studied systematically with the eyes adapted to monochromatic rays of varying wavelengths.

1. All of the accommodation curves obtained were of duplex nature, being divided into two sections by an inflexion at about 50–80 msec. in RC.

2. Hill's λ-log-radiance (I) curves were obtained separately for both cases of the time-constants (RC) shorter and longer than 50 msec. Both families of the curves for both cases of RC, were different not only in shape but also in arrangement order of the curves depending on the wavelength of the adapting light.

3. Hill's λ has a parallel relation to spectral luminosity of the adapting light, when the adaptng colored light was of equal energy.

4. It was found that the electric threshold (V) can be expressed by a simple formula as a function of the Weber fraction (W) in brightness discrimination and the intensity (I) of the adapting light, as follows,

$$V=A\ (\log\ W^{p}+\log\ I)+B,$$

where A and B are constants and p is the parameter which relates to the time-constant of the stimulating current.

5. The relation between the accommodation constant (Hill's λ) and the intensity of the adapting light, was deduced mathematically from the formula stated above, and it was indicated that the Hill's λ is inversely proportional to the Weber fraction in brightness discrimination.

I wish to express my cordial thanks to Prof. T. Mita who has given me

valuable advice throughout the experiments and many constructive criticisms in preparing this paper, and also to Dr. A. Akihama for his helpful assistance throughout this work.

References

1) Sato, M., Jap. J. Physiol., 1958, **8**, 1.
2) Steinhardt, S., J. Gen. Physiol., 1936, **20**, 185.
3) Blackwell, H. R., J. Opt. Soc. Amer., 1946, **36**, 624.
4) Hill, A. V., Proc. Roy. Soc., 1936, **119**, 305.
5) Sato., M, Suzuki, T. & Akihama, A., Jap. J. Physiol., 1957, **7**, 29.
6) Yaegashi, S., Tohoku J. Exp. Med., 1958, **67**, 163.
7) Mita, T., Fujimaki, E. & Takahashi, R., Jap. J. Physiol., 1954, **4**, 115.
8) Mita, T., Fujimaki, E. & Yaegashi, S., Tohoku J. Exp. Med., 1956, **65**, 45.
9) Motokawa, K. & Iwama, K., Tohoku J. Exp. Med., 1949, **50**, 25.

The Tohoku Journal of Experimental Medicine, Vol. 69, Nos. 2-3, 1959

Peripheral Utilization of Glucose in Diabetic Patients

By

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(Received for publication, October 3, 1958)

Blood glucose is utilized in peripheral (extrahepatic) tissues. The venous blood glucose content is always less than, or at least equal to that in the arteries, except in the hepatic venous blood which contains a large amount of glucose released from the liver. This arteriovenous blood sugar difference (A-V difference or delta glucose) is believed to indicate the utilization of glucose in peripheral tissues. (By utilization it is here meant the combined mechanism of oxidation and storage).

The A-V difference becomes greater after injection of glucagon[1] and is decreased by adrenaline[2]. The former is an agent accelerating the peripheral utilization of glucose and the latter is an inhibiting one. It is reported also that the A-V difference is greater in alimentary hyperglycemic states than in fasting states[3] and this postprandial increase in A-V difference is much less marked in diabetics in comparison with normal adults[4]-[7].

The present study was undertaken to ascertain whether the peripheral utilization of glucose is in fact diminished in diabetic patients.

Experimental

Method

Five healthy adults, eleven nondiabetic patients and twenty-five diabetic patients in hospital wards served as subjects. All subjects were studied while they were at rest after overnight fasting. Venous blood samples were obtained by antecubital venipuncture without stasis, and almost simultaneously, capillary blood specimens were collected by direct pipetting from an ear lobe after cutaneous puncture. Practically it is assumed that the sugar content of arterial blood is identical with that of capillary blood[8][9]. Consequently, in this paper we referred to sugar in the capillary blood as arterial blood sugar. The capillary and the venous blood samples were obtained prior to the oral administration of 50 g. glucose in 250 cc. of water and every thirty minutes thereafter for three

hours. Blood sugar estimations were made by the micromethod of Fujita and Iwatake[10]. In diabetic patients, this study was performed prior to the start of insulin treatment.

Results

C-V (capillary-venous) difference in fasting state. The C-V difference in fasting state has been reported to be less in diabetics in comparison with nondiabetics. Our results, however, indicate no statistically significant difference between these two groups. The mean fasting C-V difference was 10.3 mg% with a S.D. (Standard Deviation) of 6.7 mg% in nondiabetic group and 9.2 mg% with a S.D. of 6.4 mg% in diabetic group.

Fluctuation of C-V difference in glucose tolerance test. In all cases of test subjects, the C-V difference increased after ingestion of glucose. This rise in C-V difference occurred promptly in the nondiabetic group, reaching a maximum in one hour and it decreased gradually within one and half hour, sinking not only to, but below the initial fasting level (Figs. 1 and 2). The phase of increasing of C-V difference is a reflection that the venous curve is checked in rise earlier than the arterial and falls earlier ; the phase of decreasing C-V difference is the result of a steep-sloped arterial descending curve and a relatively flat-sloped venous one. In the diabetic group, on the other hand, the riese of C-V difference occurred slowly, reaching a peak level at one or one and half hours as seen in the same figures. It has been believed that the rise of C-V difference is less in diabetics in comparison with nondiabetics and this is one of the characteristic findings of diabetes mellitus. Our results, however, do not support this view. The mean maximum C-V difference was 26.3 mg% with a S.D. of 11.0 mg% in the nondiabetic group, and it was 28.9 mg% with a S.D. of 13.2 mg% in the diabetic group. No significant difference was found statistically between them. A statistical calculation of C-V difference at capillary peak values also indicates no significant difference between them, i.e., the mean value of the diabetic group was 18.4 mg% (S.D., 14.3 mg%) and that of the nondiabetic group was 20.6 mg.% (S.D, 13.6 mg%). The presence of a negative C-V difference after glucose ingestion was observed in this study, as reported by other workers (Table I, Nos. 17, 19, 20, 29, 30, 35, 37). A negative C-V difference, however, was not obtained in fasting state except in a case to be described below (Fig. 5).

Utilization index. The concept of an assimilation index was introduced by Somogyi[2][14]. His index is obtained by the formula Σd_i (d=C-V difference). Negative increments and their corresponding C-V differences were omitted from the calculation ; for example, A.I.$=d_1+d_2+d_3$ in Fig. 3, where the hyperglycemia continued until time 3.[1][14] His index is meant to grow larger, the greater the assimilation of glucose. This calculation

TABLE I

Case No.	Age Sex	Capillary blood sugar (mg %) Fasting	30	60	90	120	150	180 min.	C-V difference (mg %) Fasting	30	60	90	120	150	180 min.
	Nondiabetic group														
1	28 M	118	177	146	123	88	102	98	16	23	6	35	0	31	2
2	30 M	92	127	128	102	92	88	97	4	0	10	12	11	11	16
3	23 M	120	146	120	107	107	102	114	8	26	33	27	20	3	4
4	43 M	106	160	123	92	77	81	87	2	30	16	15	13	8	2
5	29 M	102	142	127	96	96	92	94	8	10	28	15	4	5	4
6	13 M	111	193	139	118	109	101	96	5	46	28	6	0	1	2
7	46 M	99	127	142	123	106	94	99	14	11	43	24	7	9	3
8	16 M	123	144	133	121	120	106	109	17	4	15	9	11	0	0
9	46 M	120	155	159	155	155	155	143	7	7	0	7	9	9	4
10	21 M	111	149	161	107	81	80	102	24	9	33	0	3	12	22
11	69 F	128	160	198	184	153	128	108	20	7	4	31	7	0	8
12	52 M	107	135	154	137	104	83	83	8	14	12	16	16	2	3
13	37 M	109	130	139	123	96	85	83	3	7	16	7	4	0	0
14	44 M	102	153	90	90	88	84	90	3	30	0	5	5	0	5
15	50 F	120	177	179	97	80	90	85	23	31	25	19	9	0	2
16	48 M	118	160	165	158	135	97	85	2	6	12	11	0	14	0
	Diabetic group														
17	57 M	121	198	222	222	170	118	96	7	45	29	28	-14	-21	6
18	60 M	146	194	215	191	114	100	114	11	10	38	45	3	20	27
19	50 M	137	203	259	278	154	217	182	7	5	18	24	23	5	- 7
20	43 F	128	191	227	210	181	170	121	7	3	20	29	14	28	- 9
21	50 M	167	299	337	254	247	201	184	9	4	10	15	8	34	17
22	43 M	132	222	261	186	137	102	94	11	22	28	35	21	10	13
23	47 M	133	222	247	181	135	109	94	8	7	44	42	24	19	7
24	62 F	153	198	292	334	228	204	156	5	10	32	46	6	16	0
25	44 M	174	232	266	256	222	168	111	25	4	2	16	0	6	4
26	57 M	214	278	298	274	256	212	170	6	22	4	4	10	4	0
27	43 M	160	194	212	232	240	208	174	7	0	0	0	0	4	8
28	38 M	160	218	274	274	264	214	174	7	10	4	18	8	16	8
29	60 M	187	248	309	325	280	236	215	8	12	8	33	0	1	- 7
30	54 M	324	384	506	508	506	432	401	0	-16	6	2	0	-18	0
31	44 F	266	288	350	388	388	382	334	8	24	24	30	24	10	12
32	35 F	240	358	438	440	368	316	270	6	22	42	24	6	8	4
33	57 F	288	326	406	438	378	348	316	26	6	38	24	6	12	18
34	61 M	326	424	484	504	490	444	430	0	14	30	10	12	0	0
35	55 M	214	292	392	396	350	298	254	6	0	20	34	-12	-14	-16
36	48 M	254	342	330	306	288	274	270	8	6	14	8	4	0	6
37	55 M	270	330	420	420	358	274	254	14	38	0	20	50	-20	0
38	54 M	228	284	330	378	330	302	260	0	6	0	4	0	0	0
39	42 F	246	350	382	374	334	330	288	4	6	0	0	4	8	8
40	47 F	256	372	406	452	388	372	344	14	32	20	28	20	18	14
41	49 M	334	434	466	452	396	382	354	28	10	14	14	14	34	32

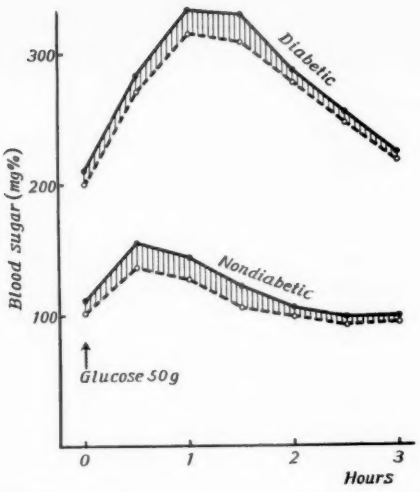

Fig. 1. Mean glucose tolerance curves. The upper is the data for diabetic group and the lower is that for nondiabetic group. The solid lines represent the capillary blood sugar curves and the broken lines represent the venous ones.

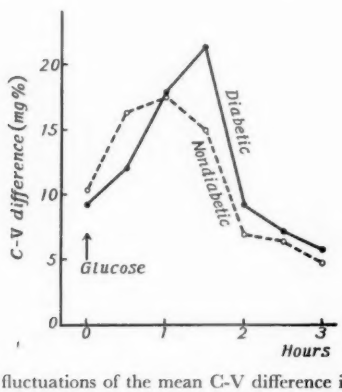

Fig. 2. The fluctuations of the mean C-V difference induced by glucose ingestion. The solid line represents the mean change of diabetic group and the broken line represents that of nondiabetic group.

was adapted to our series. The mean assimilation index of the nondiabetic group was 45.1 mg% with a S.D. of 17.9 mg% and that of the diabetic group was 70.0 mg% with a S.D. of 42.5 mg%. A statistical calculation indicated the presence of a significant difference between these groups. Our results, therefore, revealed that the assimilation index of

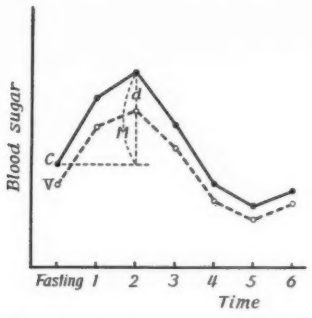

Fig. 3. C, capillary blood sugar ; V, venous blood sugar ; d, C-V difference ; M, magnitude of hyperglycemia.

diabetics is greater than that of nondiabetics ; that is, the assimilation of glucose is better in diabetics than in nondiabetics. This is a finding contradictory to present concepts. Hence, we applied a new utilization index to the data. Our utilization index during *n* hours is obtained by the formula $\sum_{i=F}^{n} d_i$ (our index consists of the values both in the hyperglycemic and hypoglycemic phase ; in other words, in our index, negative increments and their corresponding C-V differences are not omitted from the calculation). The reason of our application of such a formula is due to the fact that the utilization of glucose in peripheral tissues is dealt with not only in an alimentary hyperglycemic phase, but also in a postalimentary hypoglycemic phase, although the rate of glucose utilization may be different in the two. The mean utilization index was 78.5 mg% (S.D., 26.2 mg%) for the nondiabetic subjects and 82.7 mg% (S.D., 48.8 mg%) for the diabetic group. A statistical calculation failed to find a significant difference between these two mean values. This means that the amount of glucose utilized in peripheral tissues is equal in diabetics and nondiabetics.

Utilization rate. Somogyi introduced the term " relative assimilation index " to compare the uptakes of glucose in the presence of different capillary blood sugar levels. His index is calculated according to the formula $\frac{\Sigma d_i}{\Sigma M_i} \times 100$ in Fig. 3. We attempted to get an index representing the ratio of utilized sugar to the whole blood sugar, i.e. a ratio of the amount of delta glucose to whole amount of blood glucose. For this purpose, it seems better to compare the area constructed by the capillary blood sugar curve and the venous blood sugar curve with the area delineated by the capillary blood sugar curve and the zero line. We applied the following formula to calculate this value :

$$\text{U.R.} = \frac{\sum_{i=F}^{n} d_i}{\sum_{i=F}^{n} C_i} \times 100 \ (\%)$$

The average utilization rates, obtained in this way, were 9.8% (S.D., 3.5%) for nondiabetics and 4.9% (S.D., 3.7%) for diabetics, respectively. This result, in which the utilization rate is significantly greater in nondiabetics than in diabetics, indicates the decrease of utilization power in diabetic subjects. The relationship between the utilization rate and the fasting blood sugar is depicted in Fig. 4. Roughly speaking, the utilization rate becomes less with the increase of fasting blood sugar.

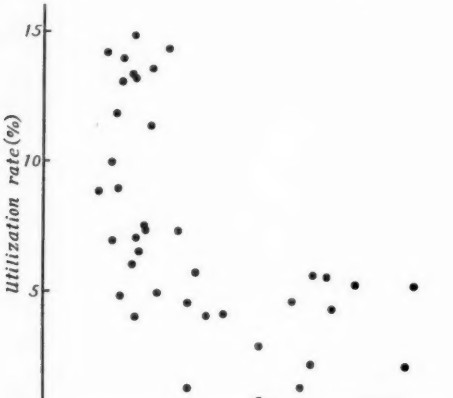

Fig. 4. The relationship of the fasting blood sugar and the utilization rate (see text).

To simplify the calculation of the utilization rate, we attempted also to calculate the ratio of the peak of the capillary blood sugar curve and the C-V difference at this peak level. This utilization rate at peak level is almost proportional to the utilization rate described above.

Discussion

It has been reported by many workers that the C-V difference is less in diabetics in comparison with nondiabetics and this difference becomes more prominent in alimentary hyperglycemia[4)-7)]. However, the present study and that of Cavett and Seljeskog[15)] disagree with this concept. Cavett and Seljeskog thought the cause of their disagreement with other workers

to be due to their diabetic patients being well managed and having received insulin the day before the test. In our series there was no case of diabetic coma or precoma although the test was performed before the start of insulin treatment. The discrepancy of our results with the others' might originate partly in the conditions of the test subjects. Another important cause of the discrepancy might be that the great majority of experiments of the other workers were performed usually with smaller numbers of test subjects and the blood specimens were collected less frequently. Rabinowitch[6], obtained blood samples at fasting state and at the half hour after the ingestion of 25 g. of glucose, and postulated a lesser increase in C-V difference of diabetic patients in alimentary hyperglycemia. If we had collected the blood specimens only at such times, we should have obtained similar results as Rabinowitch, because the C-V difference of our diabetic series at half hour is far less than that of nondiabetics even though this relation is reversed at one and half hours as shown in Fig. 2. We postulate from the present study that no significant difference of C-V difference is found between nondiabetics and diabetics in the fasting state or during the alimentary hyperglycemia.

Many investigators have confirmed the existence of negative C-V differences after the ingestion of glucose[4)5)6)12)13)], even though it was strongly denied by Somogyi whose studies were performed usually on healthy adults[16]. In the present study, negative C-V difference values were observed in the diabetic group after glucose ingestion. Griffiths[12] explained this negative C-V difference as the result of glucose, absorbed from the intestine, being held temporarily by tissues in the free state, to be liberated into the blood stream later for distribution. Recently Gray and DeLuca postulated a similar concept[17]. Friedenson and his co-workers[13] thought that under certain conditions, some part of the carbohydrate in tissues can be mobilized into the blood. We do not agree with these concepts. A 46 year-old obese man was admitted to our Clinic complaining of sudden onsets of attacks of weakness, fatiguability and sweating at midnight. His glucose tolerance curve was diabetic with positive urine sugar and his capillary blood sugar values including the fasting one were always lower than the venous (Fig. 5). The glucose tolerance test was repeated one week later and the same result was obtained. If these blood sugar values represented true sugar, glucose must be liberated or pushed out from muscle tissues as the liver. Such a reversal of the normal sugar current is incompatible with our knowledge of carbohydrate metabolism. It is possible that an increase of nonfermentable reducing substances in venous blood may produce an apparent negative C-V difference. A remarkable increase in the nonfermentable reducing substances after glucose ingestion was observed by Mosenthal[18].

Fig. 5. 46 year-old male. Glucose tolerance test. The solid line represents the capillary blood sugar, the broken line the venous blood sugar.

In the present study, the amount of sugar utilized in peripheral tissues of diabetics (utilization index) is equal to that of nondiabetics, although the utilization rate is extremely low in diabetics. This fact suggest that an equal quantity of sugar is utilized in the tissues of both diabetics and nondiabetics, but the diabetic condition requires high glycemia to utilize this sugar. Similar findings were obtained by Soskin and Levine in experimental diabetic animals[19]. Bondy and his co-workers found by analysis of arterial and hepatic venous blood that diabetic patients utilized the normal quantity of glucose in postabsorptive state[20]. This relation is likened to a water-mill; the water level is the blood sugar content, the number of revolutions of the wheel or the work load is the utilization of glucose and the ratio of the work load to the water level is the utilization rate. An outworn and inefficacious water-mill needs a stronger water stream, in other words, a higher water level to revolve the wheel than a new efficacious one. The abnormally high level of blood sugar in diabetics, therefore, is not a cause, but a result of adaptation to their inefficient carbohydrate metabolism.

Our results seem to support not only the Underutilization Theory but the Overproduction Theory of diabetes. The hyperglycemic state, in which as much sugar is utilized as in the normal state and furthermore, a large amount of blood sugar is excreted by the kidney without utilized, cannot appear without sugar production exceeding the normal range. The overproduction of sugar, however, may not be a cause of diabetes, but an adaptation phenomenon in response to the demand for glucose in peripheral tissues. The overproduction of sugar in diabetic patients was confirmed by Bondy and his colleagues[20], and the recent investigation of Ashmore and his co-workers[21] which showed the increase in glucose-6-

phosphatase in alloxan diabetes rat liver. Increases of hepatic glucose output were observed by Bearn and his co-workers[22] in diabetic patients and by Crandall and Lipscomb[23] in diabetic dogs, too, but they concluded that the increase was not statistically significant in comparison with the normal group.

If the peripheral utilization of sugar is extremely diminished and the blood sugar fails to rise to a sufficient high level to utilize a normal amount of sugar, fat and protein are compulsorily decomposed. The oxidation of these intermediate metabolites in tricarboxylic cycle, however, is blocked by the deficiency of oxalacetic acid which is partly supplied by pyruvic acid and joins with acetyl fragments to form citrate. Then superfluous Acetyl CoA condenses to Acetoacetyl CoA and a large amount of acetoacetic acid is formed. This deranged adaptation of the body to the diminished utilization of carbohydrate is the cause of diabetic ketosis.

Summary

Simultaneous estimations of capillary and venous blood sugar were made in twenty five diabetic patients and sixteen nondiabetic adults during glucose tolerance test. No significant difference in fasting and alimentary hyperglycemic C-V difference was found between these two groups. The utilization index, which denotes the sum of C-V difference in each hour, was almost equal in both groups, but the utilization rate, represented by the ratio of the sum of C-V differences and the sum of capillary blood sugar values in each time, was small in the diabetic group in comparison with the nondiabetic group.

These results suggest that the amount of glucose utilized in peripheral tissues is equal both in diabetics and nondiabetics, but the diabetic condition requires high blood sugar level to utilize a normal amount of glucose.

References

1) Van Itallie, Morgan & Dotti, J. Clin. Endocrinol., 1955, **15**, 28.
2) Somogyi, J. Biol. Chem., 1950, **186**, 513.
3) Somogyi, *ibid.*, 1948, **174**, 189.
4) Bose, Ind. J. Med. Res., 1935, **23**, 1.
5) Blitstein, Arch. Mal. Appar. Digest., 1939, **29**, 268.
6) Rabinowitch, Brit. J. Exper. Path., 1927, **8**, 76.
7) Lachnit, Reinhardt & Eberhartinger, Wien. Zschr. inn. Med., 1954, **35**, 15.
8) Foster, J. Biol. Chem., 1923, **55**, 291.
9) Langner & Fies, Am. J. Clin. Path., 1942, **12**, 559.
10) Fujita & Iwatake, Biochem. Zschr., 1931, **242**, 43.
11) Lawrence, Brit. Med. J., 1924, **1**, 516.
12) Griffiths, Clin. Science, 1938, **3**, 91.
13) Friedenson, Rosenbaum, Thalheimer & Peters, J. Biol. Chem., 1928, **80**, 269.
14) Somogyi, J. Biol. Chem., 1949, **179**, 1289.

15) Cavett & Seljeskog, J. Lab. & Clin. Med., 1933, **18**, 1103.
16) Somogyi, J. Biol. Chem., 1949, **179**, 217.
17) Gray & DeLuca, Canad. J. Biol. & Physiol., 1956, **34**, 763.
18) Mosenthal, Med. Clin. North America, 1947, March, 299.
19) Soskin & Levine, Am. J. Physiol., 1937, **120**, 761.
20) Bondy, Bloom, Whitner & Farrar, J. Clin. Invest., 1949, **28**, 1126.
21) Ashmore, Hastings & Nesbett, Proc. Nat. Acad. Science, 1954, **40**, 673.
22) Bearn, Billing & Sherlock, Lancet, 1951, **261**, 698.
23) Crandall & Lipscomb, Am. J. Physiol., 1947, **148**, 312.

The Tohoku Journal of Experimental Medicine, Vol. 69, Nos. 2-3, 1959

Application of the Sinusoidal Alternating Current at Frequency of 3 cps. for Measuring Fatigue

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(Received for publication, November 11, 1958)

Introduction

Motokawa & Iwama[1] have first reported that the electrical excitability of the eye is remarkably sensitive to the increase or decrease in oxygen content of the blood. Thereafter, Motokawa & Suzuki[2][3] showed that this phenomenon can be applied to the measurement of fatigue. They took as an index of fatigue a measure ΔS, which represents the difference between the threshold (S_1) for appearance and that (S_2) for disappearance of the flickering phosphenes elicited by the repetitive rectangular currents or the sinusoidal alternating currents (A.C.) at 20 cps. in frequency. As to the essencial factors of ΔS, Mita, Abe & Byonshik[4] reported that the ΔS can be analized into two factors, namely the intensity factor (electric threshold) and the time factor (reaction time). Lately, it was elucidated by Mita, Fujimaki & Takahashi[5] that the stimulating effect of the eye with A.C. at frequeny of 3 cps. is quite different from that with A.C. at frequency of 20 cps. Above all, noticeable is the fact that in the case for 3 cps., the relation between the eletric threshold and the intensity of illumination is quite similar to the Ferry-Porter's law concerning the critical fusion frequency (C.F.F.), which is not the case with 20 cps.

On the other hand, C.F.F. has long been known to be used as an indicator of fatigue. From these points of view, we attempted to examine whether A.C. at frequeny of 3 cps. is also applicable to the method for measuring fatigue as well as C.F.F. and A.C. of 20 cps. (Motokawa's method).

Experimental

Methods

A silver plate of 2×3 cm. in size was used as a stimulating electrode

and was inserted between upper lip and gum. Another large silver plate, as an indfferent electrode,was placed under the right sole, being immersed into 1% saline. Fig. 1 shows the block diagram of arrangement for measuring fatigue.

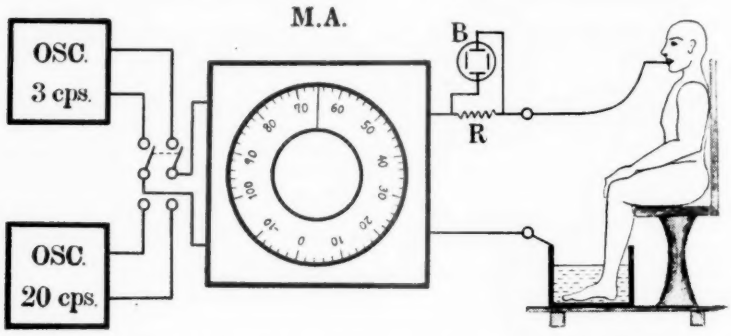

Fig. 1. Block diagram of the arrangement for measuring fatigue. M.A. is Motokawa's apparatus for measuring fatigue, (manufactured by Tōa Dempa Kōgyō Co., Ltd.). B is a cathode ray oscilloscope. O.S.C. is a RC type oscillator.

We could measure two sorts of electric thresholds for both stimulating currents of 3 cps. and 20 cps. in turn, by using a commutator as shown in Fig. 1. The stimulating current was increased from zero at a constant rate of 0.04 mA per second to determine the threshold (S_1) for appearance of flickering phosphene, then the threshold (S_2) for disappearance of flicker was determined by decreasing the current from a sufficiently high level at the same rate as above. The average value of the threshold was calculated from ten different measurements of the same threshold. The difference of both sorts of thresholds, namely S_1–S_2, was taken as an indicator of fatigue and it was denoted by $\Delta S(I)$. At the same time, the impedance (Imp) of the body between both electrodes was measured, therefore the $\Delta S(I)$ in mA could be expressed in volt ($\Delta S(V)$). All data were taken from 3 well-trained subjects for perception of the electrical phosphene and 12 untrained ones.

The exercise we employed was to ascend and descend the stairs, of which one step was 27 cm. in height at a 36° inclination angle, at a rate of one step per second, holding overhead a bar-bell of 24 kg., or carrying a load of 10 kg. on the subject's back. As will be described later, the electric threshold of the eye for 3 cps. is the lowest in darkness, so that we were used to determine the thresholds with the dark-adapted eyes. This was also convenient for keeping constant the adaptation state of the eye during the measurement. Accordingly, as soon as the subject ended his exercise,

he had to enter into a dark room to take a seat in an armchair, putting the electrodes on his parts mentioned above. As the electric threshold of the eye for 3 cps. reached the level of the fully dark-adapted eye practically within one minute after removal of the light, as reported by Mita, Fujimaki & Yaegashi[6], it was not necessary to stay in the dark the same length of time as in the cases for 20 cps. or for the adequate stimuli before beginning the measurements. In fact, we commenced immediately after entering the dark.

Results

a) Dependence of the electric threshold on the ambient illumination

In order to overlook the dependence of the electric threshold on the ambient illumination, both electric thresholds of the dark-adapted eye and the light-adapted one were compared with each other in both cases of A.C. at 3 cps. and 20 cps. The data obtained were plotted in Fig. 2. As can be seen in Fig. 2, the electric threshold for 20 cps. decreases with increase in intensity from dark to 7.8 lux, while that for 3 cps. increases rapidly with increase in intensity within this range. These findings coincide remarkably with the results reported by Mita, Fujimaki & Takahashi[5] and Mita, Fujimaki & Yaegashi[6]. Fig. 2 shows that in both cases of 3 cps. and 20 cps., the lower the values of S_1 and S_2 are, the smaller the value of ΔS becomes respectively. Thus, all the measurements of the electric thresholds were always made in a dark room throughout this experiment, as described before.

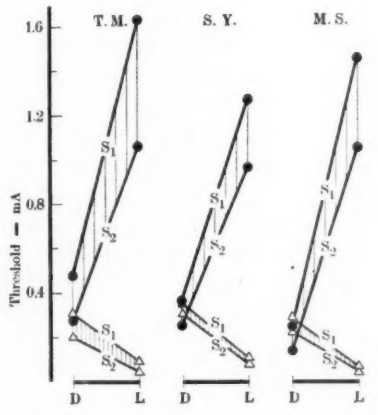

Fig. 2. Dependence of S_1, S_2 and ΔS on the ambient illumination. D and L mean the dark and light adaptation (7.8 lux at the corneal surface) respectively. ●——●: The data for 3 cps., △——△: The data for 20 cps.

b) Variation in the value of $\Delta S(I)$ for 3 cps. during a day

Three of the authors served as the subjects and $\Delta S(I)$ was measured during about 10 hour's duty. At the same time, the body impedance (Imp) between both electrodes was measured. The data obtained are shown in Fig. 3. As can be seen in Fig. 3, all the $\Delta S(I)$-curves showed two maxima at 12–1 p.m. and a minimum at 2–3 p.m. If these results are compared with those reported by Abe & Byonshik[7] and Motokawa & Umetsu[8], our fatigue curves are found to be similar in shape with those obtained by them. But the

maxima and the minimum on our fatigue curves appear about one hour later than those on their curves. This retardation may be due to the circumstance that all our subjects took their lunch at 1.00–1.30 p.m., while their subjects did at noon. On the other hand, the impedance did not show so remarkable a variation during a day as in the case of $\Delta S(I)$, but it was noteworthy that all the impedance curves showed a systematic variation, that is, a slight crest was found regularly at 4–6 p.m. 1–2 hours later than a trough at 2–3 p.m. on the $\Delta S(I)$-curve.

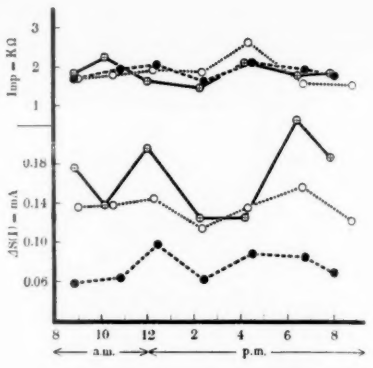

Fig. 3. Variations in the values of ΔS (I) and impedance (Imp) during 10 hour's duty. ●---●: Subj. S.Y., ○·····○: Subj. M.S., ⊕——⊕: Subj. T.M.

c) Test of fatigue by exercise

The amount of work of 500–2800 kg-m was performed by means of the exercise mentioned above, and the variations in $\Delta S(I)$, $\Delta S(V)$, Imp for 3 cps. and C.F.F. were recorded in this course of the experiment. The data obtained for 4 well trained subjects for perception of the electrical phosphene are shown in Fig. 4.

As can be seen in Fig. 4, both values of $\Delta S(I)$ and $\Delta S(V)$ increase with increase in the severity of the work, while both values of Imp and C.F.F. decrease in the reverse direction. These findings for $\Delta S(I)$ and $\Delta S(V)$ for 3 cps. are well consistent with those results for 20 cps. which were reported by Suzuki[9] and the others. The findings for C.F.F. are also well consistent with those reported by many investigators[10]. If we compare these fatigue curves between female and male subjects, the woman's fatigue seems to be greater than the man's for the same amount of work.

It is worth to note that even at 40 minutes after the end of the exercise amounting to 2660 kg-m, the value of impedance still remained at a lower value, while the values of $\Delta S(I)$, $\Delta S(V)$ and C.F.F. had already returned to their initial values at that time. Many investigators mentioned above have reported that during and after the severe exercise, the skin impedance under the stimulating electrodes decreases with perspiration. In all cases of their experiments, the stimulating electrodes were placed on the skin parts of the head. In our case, however, the stimulating electrode was inserted between upper lip and gum, so that here is no influence of the perspiration. Although the skin impedance of the foot, including the sole, under the indifferent electrode is usually below 500 Ω, it is possible to be influenced in some special cases by both the mental and thermal

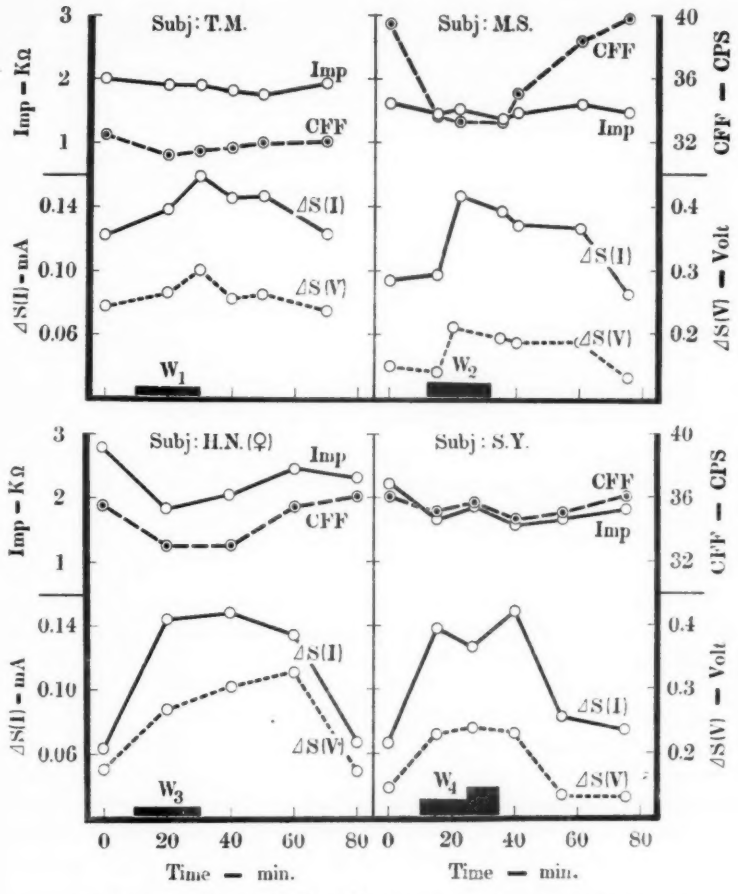

Fig. 4. Variations in $\Delta S(I)$, $\Delta S(V)$, impedance (Imp) and C.F.F due to the varying amounts of work. W_1, W_2, W_3 and W_4 express the amounts of work of 1410, 1760, 1410 and 2660 kg-m respectively.

sweatings. Accordingly, the cause of decrease in the impedance during and after the severe exercise only may be related to either the sweatings or some other factor such as secretion at the mucous membrane of the oral cavity.

The percent increase in $\Delta S(I)$, $\Delta S(V)$ and Imp versus the varying amounts of work was calculated by the following formula in each case of 15 healthy adults; $(Y-Y_0/Y_0)\times 100$, where Y_0 and Y mean the values of ΔS or Imp before and after the exercises respectively. These data were

plotted as ordinates against log (Work) as abscissae in Fig. 5. As can be seen in Fig. 5, the percent increase in $\Delta S(I)$ shows a linear relation to log (Work), while that of Imp is inversely proportional to log (Work). Therefore, the percent increase in $\Delta S(I)$ due to the exercise is always greater than that of $\Delta S(V)$, which is the product of the Imp and $\Delta S(I)$. If the variance of the data for $\Delta S(I)$ is compared with that for $\Delta S(V)$, the former is greater than the latter. From these facts it can be concluded that the $\Delta S(I)$ is more sensitive than $\Delta S(V)$ as an indicator of fatigue, but the standard deviation of the data for $\Delta S(I)$ is larger than that for ΔS (V).

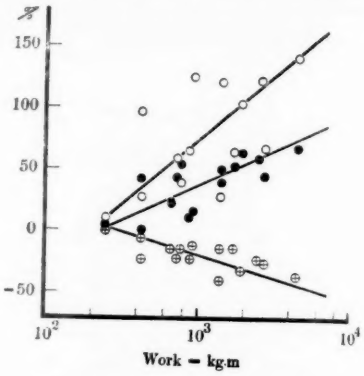

Fig. 5. Relation of the percent increase in $\Delta S(I)$, $\Delta S(V)$ and Imp. to the amount of work. ○: The data for $\Delta S(I)$, ●: $\Delta S(V)$ and ⊕: Imp.

Discussion

From the results mentioned above, it was proved that ΔS determined by A.C. at 3 cps. in frequency is also applicable as an indicator for measuring fatigue in the same manner as that by A.C. at 20 cps., which was employed by Motokawa *et al.* Comparing our method with Motokawa's, we will discussed the relative merits of both methods in the practical application.

a) In Motokawa's method, the stimulating electrodes of the eye are placed on the skin near the eyeball, so that the electric threshold of the eye is influenced more or less by perspiration at the skin under the electrodes. But, as the stimulating electrode in our method is inserted between upper lip and gum, the influence of sweating with exercise needs scarcely be considered.

b) The determinations of the S_1 and S_2 in Motokawa's method must be done under a certain moderate illumination; while in our method, those determinations must be performed in a dark room, or the light must be shut off completely from the subject's eyes. From this viewpoint, our method seems to be somewhat more troublesome than Motokawa's, but it is actually rather convenient, because we need not take into account any influence of either the ambient illumination or the time required for adaptation on the electric threshold of the eyes during fatigue measurements.

c) The electrical phosphenes evoked by A.C. of 20 cps. come into sight at first in the periphery of the visual field as a flickering light. On the other hand, the electrical phosphenes elicited by A.C. of 3 cps. can be

seen most clearly in the center of vision, like the adequate stimuli pulsating with a slow rhythm of 3 cps. Accordingly, the electrical phosphenes for 3 cps. can be perceived more easily than those for 20 cps., especially easily for the untrained subjects. However, as shown in Fig. 2, the values of the electric threshold of the dark-adapted eye for 3 cps. are usually higher than the values for 20 cps. which are obtained from the eyes adapted to the moderate intensity of illumination. This point is the only demerit in our method.

Summary

Using the sinusoidal alternating current (A.C.) at 3 cps. in frequency as the stimuli of the eye, we determined the threshold (S_1) for appearance of the flickering phosphene, and the threshold(S_2) for disappearance of it. Calculating the difference of both thresholds, namely $S_1-S_2=\Delta S$, we investigated the applicability of this measure (ΔS) as an index for measuring fatigue. At the same time, Motokawa's ΔS for 20 cps., C.F.F. and the body impedance were measured for comparison with our measure (ΔS for 3 cps.).

1. Each value of S_1, S_2 and ΔS for 3 cps. was the smallest in darkness, while that for 20 cps. was the smallest under moderate illumination.

2. In the course of 10 hour's duty, the values of ΔS showed two maxima before and after lunch respectively. This finding is consistent with the result reported by Abe & Byonshik. Impedance did not show so remarkable a variation in the same course as the former.

3. When the work of 500–2800 kg-m was performed by the 15 healthy subjects, the temporal variations in the values of ΔS, impedance and C.F.F. were investigated. The value of ΔS increased with increase in the amount of work, while the values of C.F.F. and the body impedance decreased with increase in load. These findings are in conformity with the results studied by Motokawa's original method.

4. From the results mentioned above, it was concluded that the A.C. at 3 cps. can be useful for measuring fatigue as did the A.C. at 20 cps. by Motokawa *et al.* And the merits and demerits of our method on the practical application were discussed in comparison with Motokawa's method.

References

1) Motokawa, K. & Iwama, K., Tohoku J. Exp. Med., 1949, **50**, 319.
2) Motokawa, K. & Suzuki, K., Jap. Med. J., 1948, **1**, 200.
3) Motokawa, K. & Suzuki, K., Nisshin Igaku (Jap.), 1948, **35**, 523.
4) Mita, T., Abe, Z. & Byonshik, T., Tohoku J. Exp. Med., 1951, **54**, 45.
5) Mita, T., Fujimaki, E. & Takahashi, R., Jap. J. Physiol., 1954, **4**, 115.
6) Mita, T., Fujimaki, E. & Yaegashi, S., Tohoku J. Exp. Med., 1956, **65**, 45.

7) Abe, Z. & Byonshik, T., Tohoku Ig. Z. (Jap.), 1950, **45**, 49.
8) Motokawa, K. & Umetsu, J., Rōdōno Kagaku (Jap.), 1957, **12**, 28.
9) Suzuki, K., Tohoku J. Exp. Med., 1950, **52**, 1.
10) Sangyō Hirō Iinkai, Rōdōno Kagaku (Jap.), 1952, **7**, Suppl. 1.

The Tohoku Journal of Experimental Medicine, Vol. 69, Nos. 2-3, 1959

Effects of BZ 55 and DBI on Blood ATP, Inorganic Phosphorus and Capillary-Venous Blood Sugar Difference

By

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(Received for publication, November 27, 1958)

One of the recent brilliant progress in the treatment of diabetes mellitus is the discovery of effective oral hypoglycemic agents.[1)-3)] Ito reported the effect of insulin on blood ATP (adenosine triphosphate) level in diabetic patients. He found that insulin lowered the blood inorganic phosphorus level and raised the blood ATP content in diabetics as well as in non-diabetics, but these changes were significantly greater in healthy adults than in diabetic patients.[4)] In the present study we attempted to ascertain what changes would be produced by the oral administration of BZ 55 (N_1-Sulphanilyl-N_2-*n*-butylcarbamide) and DBI (N'-beta-phenethylformamidinyliminourea) in diabetic patients and healthy adults.

Experimental

Method

Nine diabetic inpatients and eight healthy volunteers were used as test subjects. After an overnight fast, the test subjects ingested 1 g. of BZ 55 or 100 mg. of DBI and the blood specimens were obtained by the antecubital venipuncture prior to and every hour after the ingestion of these drugs for the blood ATP, inorganic phosphorus and venous blood sugar estimations. The other blood specimens were collected from the ear lobe by direct pipetting for capillary (arterial) blood sugar determinations. The methods of determination of blood ATP, blood inorganic phosphorus and blood sugar were the same as described elsewhere.[5)] The test were performed before the institution of insulin treatment in diabetic group.

Results

Blood ATP. The oral administration of 1 g. of BZ 55 produced a

decrease in blood ATP through four hours in the healthy group except one case (No. 2). In seven of the nine diabetic cases, on the contrary, the blood ATP increased definitely and this increase was prominent at the 4th hour. It was statistically computed that the maximum increase rate (in per cent) was significantly greater in diabetic group than in nondiabetic group (Fig. 1).

In the experiment on DBI, the blood ATP level increased slightly after the ingestion of this drug in four of the six diabetic patients and in five of the eight healthy adults, and there was no statistically significant

TABLE I

Blood 7 min. P, Inorg. P, Capillary Blood Sugar and C-V Difference after BZ 55 Administration

Case No.	Age Sex	Amounts of blood chemical components (mg%)					
			Before the administration	Time (hrs) after the administration			
				1	2	3	4
	Healthy group						
1	20 M	Blood 7 min. P	3.5	3.6	3.5	3.5	3.0
		Inorg. P	3.3	3.3	3.7	4.3	4.0
		Blood sugar	112	109	104	97	74
		C-V diff.	10	15	8	1	3
2	19 M	Blood 7 min. P	3.5	3.3	3.7	3.7	3.9
		Inorg. P	4.4	4.5	4.1	3.7	4.3
		Blood sugar	117	90	76	76	88
		C-V diff.	15	2	-23	-7	1
3	20 M	Blood 7 min. P	4.5	4.4	4.0	3.7	3.9
		Inorg. P	2.2	2.2	2.0	2.0	1.9
		Blood sugar	111	94	90	85	94
		C-V diff.	12	18	16	5	-13
4	22 M	Blood 7 min. P	5.5	5.0	5.1	5.3	5.5
		Inorg. P	3.0	3.0	2.8	2.9	2.9
		Blood sugar	97	90	77	83	80
		C-V diff.	-3	0	6	-2	0
5	22 M	Blood 7 min. P	5.0	4.7	4.4	5.0	5.3
		Inorg. P	3.6	3.5	2.7	2.7	2.6
		Blood sugar	96	92	94	96	85
		C-V diff.	-4	4	9	8	-2
6	23 M	Blood 7 min. P	5.6	5.1	5.4	4.2	4.2
		Inorg. P	3.1	2.7	2.1	2.4	2.5
		Blood sugar	107	94	90	92	88
		C-V diff.	5	-5	2	4	-6
7	22 M	Blood 7 min. P	4.8	4.0	4.1	4.0	4.4
		Inorg. P	3.9	4.0	3.9	3.8	3.7
		Blood sugar	109	97	77	92	94
		C-V diff.	2	-3	4	0	6
8	22 M	Blood 7 min. P	4.5	3.9	4.4	4.6	4.4
		Inorg. P	3.9	3.6	3.5	3.4	3.5
		Blood sugar	107	109	92	92	92
		C-V diff.	11	9	0	0	7

Diabetic group

9	43 F	Blood 7 min. P	3.4	3.3	3.7	3.9	3.5
		Inorg. P	4.8	4.8	4.3	4.2	4.0
		Blood sugar	288	273	242	228	214
		C-V diff.	38	40	16	27	28
10	59 M	Blood 7 min. P	4.0	3.8	3.6	3.9	4.0
		Inorg. P	2.6	2.6	2.6	2.6	2.5
		Blood sugar	182	160	144	125	121
		C-V diff.	-4	-6	-2	-7	-2
11	60 M	Blood 7 min. P	3.9	4.1	4.1	4.2	4.3
		Inorg. P	4.3	4.2	4.2	4.2	4.2
		Blood sugar	135	125	100	112	112
		C-V diff.	5	4	3	6	6
12	46 F	Blood 7 min. P	2.9	3.2	3.1	3.7	3.8
		Inorg. P	4.5	4.5	4.7	4.0	3.6
		Blood sugar	274	261	210	174	140
		C-V diff.	20	25	19	-3	13
13	47 M	Blood 7 min. P	3.2	3.6	3.5	3.9	3.7
		Inorg. P	3.9	3.6	3.7	3.6	3.7
		Blood sugar	139	118	111	92	85
		C-V diff.	4	-5	2	-2	-3
14	55 M	Blood 7 min. P	4.3	4.5	4.2	4.5	4.8
		Inorg. P	4.9	4.5	5.0	5.0	4.6
		Blood sugar	142	107	111	111	112
		C-V diff.	3	1	2	4	5
15	63 F	Blood 7 min. P	4.5	3.9	4.4	4.0	4.7
		Inorg. P	3.8	3.7	3.6	3.6	3.1
		Blood sugar	179	149	137	112	102
		C-V diff.	28	5	-5	10	0
16	73 F	Blood 7 min. P	3.8	2.6	4.8	3.8	3.9
		Inorg. P	3.4	3.1	1.5	1.9	3.0
		Blood sugar	123	104	87	87	100
		C-V diff.	2	-2	-3	-5	6
17	60 M	Blood 7 min. P	4.8	5.2	5.1	5.1	5.1
		Inorg. P	2.0	1.9	1.9	2.0	2.5
		Blood sugar	154	139	142	133	127
		C-V diff.	1	2	7	3	0

difference in maximum increase rate between the two groups.

Blood inorganic phosphorus. The ingestion of BZ 55 resulted usually a decrease in blood inorganic phosphorus in both groups. The statistical analysis revealed no significant difference in maximum decrease rate between these two groups.

The blood inorganic phosphorus fell after the oral administration of DBI in both groups and there was no statistically significant difference in maximum decrease rate between these two groups.

Capillary-venous blood sugar difference. The C-V difference became less or reversed in seven of the nine diabetic patients and in six of the eight healthy subjects after the ingestion of BZ 55. In a few cases, this C-V difference became greater after the administration of BZ 55.

In the experiments of the administration of DBI, the C-V difference became decreased or reversed in all cases of these subjects.

Discussion

There are several theories on the hypoglycemic action of BZ 55, i.e. 1) the acceleration of insulin secretion theory or the β-cell stimulating theory,[6] 2) the inhibition of glucagon secretion theory or the α-cell destruction theory,[7] 3) the insulinase inhibition theory,[8] 4) the theory of the inhibition of sugar output from the liver,[9][10] and so on. However, the mechanism of hypoglycemic action by this drug is not clear.

The decrease in blood inorganic phosphorus after BZ 55 administra-

Table II

Blood 7 min. P, Inorg. P, Capillary Blood Sugar and C-V Difference after DBI Administration

Case No.	Age Sex	Amounts of the blood chemical components (mg%)					
			Before the administration	Time (hrs) after the administration			
				1	2	3	4
Healthy group							
1	20 M	Blood 7 min. P	3.6	3.8	4.0	3.8	3.6
		Inorg. P	4.0	4.0	3.5	3.9	3.6
		Blood sugar	107	109	102	104	111
		C-V diff.	3	9	-9	-12	-19
2	20 M	Blood 7 min. P	3.7	4.1	3.7	3.8	3.5
		Inorg. P	4.2	3.8	3.9	3.8	4.0
		Blood sugar	102	94	86	84	89
		C-V diff.	2	9	3	-14	-9
3	19 M	Blood 7 min. P	2.9	2.6	3.7	2.7	3.5
		Inorg. P	5.2	5.2	5.0	5.0	4.5
		Blood sugar	112	96	87	99	100
		C-V diff.	3	4	-9	-12	1
4	22 M	Blood 7 min. P	5.2	5.2	5.6	5.4	5.7
		Inorg. P	3.2	3.9	3.3	3.9	4.0
		Blood sugar	116	112	118	112	116
		C-V diff.	7	0	6	3	10
5	22 M	Blood 7 min. P	5.9	6.6	6.5	6.4	6.5
		Inorg. P	4.4	4.8	3.8	4.3	4.3
		Blood sugar	100	102	99	96	100
		C-V diff.	0	5	-3	-1	0
6	23 M	Blood 7 min. P	5.6	5.3	5.2	5.6	5.5
		Inorg. P	3.6	2.9	2.5	2.2	2.4
		Blood sugar	116	112	111	107	107
		C-V diff.	4	1	5	0	1
7	22 M	Blood 7 min. P	5.7	5.8	4.5	5.0	5.2
		Inorg. P	3.4	3.0	2.4	2.8	3.2
		Blood sugar	121	116	109	107	100
		C-V diff.	7	2	9	5	0
8	22 M	Blood 7 min. P	5.0	4.9	4.6	4.9	5.1
		Inorg. P	3.0	2.8	2.5	2.8	2.9
		Blood sugar	107	108	99	106	111
		C-V diff.	7	6	2	0	5

Diabetic group

9	46 F	Blood 7 min. P	3.6	4.3	3.4	4.0	3.7
		Inorg. P	4.5	4.0	4.5	4.2	4.3
		Blood sugar	208	180	161	139	133
		C-V diff.	14	8	3	-12	-41
10	63 F	Blood 7 min. P	2.8	3.4	3.6	4.2	4.5
		Inorg. P	4.6	3.9	3.3	2.7	2.4
		Blood sugar	132	127	121	123	111
		C-V diff.	5	-1	-2	0	-9
11	59 M	Blood 7 min. P	4.4	4.1	4.0	4.2	4.1
		Inorg. P	3.3	3.6	3.5	3.3	3.0
		Blood sugar	189	187	170	160	163
		C-V diff.	-4	-4	-9	-3	-2
12	73 F	Blood 7 min. P	5.2	5.1	4.6	4.8	4.6
		Inorg. P	2.7	2.6	2.9	2.6	2.5
		Blood sugar	132	130	116	113	99
		C-V diff.	12	12	10	9	-9
13	60 M	Blood 7 min. P	4.1	4.3	4.3	4.0	4.1
		Inorg. P	2.3	2.5	2.5	2.6	2.2
		Blood sugar	248	242	229	220	200
		C-V diff.	6	-3	-26	8	6
14	26 F	Blood 7 min. P	5.6	4.6	4.8	4.9	4.7
		Inorg. P	3.1	3.7	3.6	3.7	3.9
		Blood sugar	301	274	226	205	203
		C-V diff.	21	15	-13	-17	-9

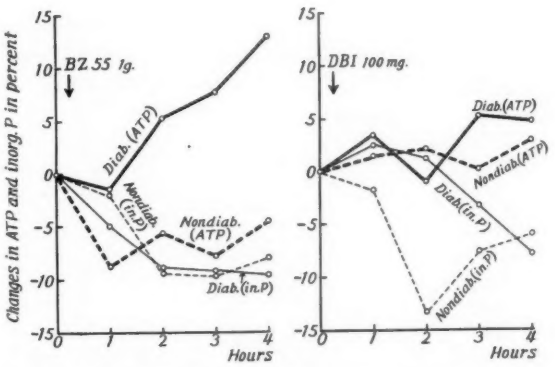

Fig. 1. Changes of blood ATP and inorganic phosphorus caused by the oral administrations of BZ 55 and DBI in per cent. The solid lines represent the average values of diabetic patients and the broken lines represent those of healthy adults.

tion and no constant effect of BZ 55 on the C-V difference were reported by some workers.[11)-16)] The present results indicate that the hypoglycemic action of BZ 55 is somewhat different from that of insulin. The C-V difference becomes greater and the blood ATP level increases significantly greater in healthy group than in diabetic group after the injection of in-

sulin, but, on the other hand, the C-V difference becomes usually smaller after BZ 55 administration and the rise of blood ATP is significantly greater in diabetics in comparison with nondiabetics. The increase in blood ATP and the decrease in blood inorganic phosphorus after BZ 55 ingestion seem to suggest that BZ 55 accelerates the metabolism of peripheral tissues, especially in diabetics.

The hypoglycemic action of DBI is known more incompletely than that of BZ 55. Unger and his co-workers speculated that DBI involved peripheral glucose utilization.[3] The decrease in blood inorganic phosphorus after DBI, obtained in this experiment, may support their speculation, but the decrease in C-V difference disagree with their concept.

Summary

The effects of BZ 55 and DBI on blood ATP, blood inorganic phosphorus and capillary-venous blood sugar difference were studied in nine diabetic patients and eight healthy adults. The oral administration of 1 g. of BZ 55 caused a decrease in blood inorganic phosphorus and C-V difference in diabetics as well as in nondiabetics. The blood ATP level, however, was raised by BZ 55 administration in diabetic group and lowered in nondiabetic group.

The oral administration of 100 mg. of DBI produced usually a slight increase in blood ATP and decreases in blood inorganic phosphorus and in C-V difference. There was no statistically significant difference in these changes between the two groups.

References

1) Franke & Fucks, Dtsch. Med. Wschr., 1955, 1449.
2) Achelis & Hardebeck, *ibid.*, 1955, 1452.
3) Unger, Freedman & Shapiro, Proc. Soc. Exper. Biol. & Med., 1957, **95**, 190.
4) Ito, Tohoku Igaku Z., (Jap.), 1957, **56**, 592.
5) Goto & Ito, Tohoku J. Exper. Med., 1957, **67**, 65.
6) Colwell, Colwell & Colwell, Metabolism, 1956, **5**, 749.
7) Loubatieres, Press. Med., 1955, **63**, 1728.
8) Mirsky, Perisutti & Diengott, Metabolism, 1956, **5**, 156.
9) Purnell, Arai, Pratt, Hlad & Elrick, *ibid.*, 1956, **5**, 778.
10) Hawkins, Ashworth & Haist, Canad. Med. Ass. J., 1956, **74**, 972.
11) Mohnike & Bibergeil, Dtsch. Med. Wschr., 1956, 900.
12) Goetz, Gilbertsen & Josephson, Metabolism, 1956, **5**, 788.
13) Hunt, Oakley & Lawrence, Brit. Med. J., 1956, 445.
14) McKenzie, Marshall, Stowers & Hunter, *ibid.*, 1956, 448.
15) Recant & Fischer, Ann. N. Y. Acad. Sci., 1957, **71**, 62.
16) Ashmore, Cahill & Earle, *ibid.*, 1957, **71**, 131.

The Tohoku Journal of Experimental Medicine, Vol. 69, Nos. 2-3, 1959

Changes of Blood ATP, Inorganic Phosphorus and Blood Sugar after Injection of Glucuronic Acid in Diabetics and Nondiabetics

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In the previous reports, we showed the latent disturbances in the production of high energy bound phosphate in diabetic patients[1)-4)] and alloxan diabetes rabbits.[5)] These disturbances are one of the most principal metabolic abnormalities in diabetes mellitus, although it is not the specific one for this disease.

It was demonstrated in our Clinic that glucuronic acid gave sometimes a significant curative effect on diabetes mellitus, and it augments the curative effect of insulin in some diabetic patients.[6)] The present study was undertaken to see what changes in blood ATP (adenosine triphosphate), blood inorganic phosphorus and blood sugar would be provoked by the administration of glucuronic acid.

Experimental

Method

Nine diabetic inpatients and seven nondiabetic adults were used in this study. After an overnight fast, 1000 mg. of sodium glucuronate (Chugai Co.) was injected intramuscularily. Blood specimens were obtained by the antecubital venipuncture prior to and every hour after the injection of glucuronic acid for blood ATP and inorganic phosphorus determinations, and the other blood specimens were collected from the ear lobe by direct pipetting for blood sugar estimation. The determinations of blood ATP were made by the same method described in the previous report except hydrolyzing in 1 *N* HCl for seven minutes.[1)] Blood sugar estimations were made by the micromethod of Fujita and Iwatake. In diabetic patients, the test was performed usually prior to the initiation of insulin treatment, and in a few cases it was done without giving insulin

only on the morning of the test.

Results

Blood ATP. The injection of sodium glucuronate caused a decrease in blood ATP at the 1st hour, in some cases at the 1st and the 2nd hours, followed an increase at the 3rd and the 4th hours. There was no statistically significant difference in maximum increase rate (in per cent) between the diabetic and the nondiabetic groups. However, comparison with nondiabetic group as seen in Fig. 1.

Blood inorganic phosphorus. The blood inorganic phosphorus decreased after the injection of glucuronic acid in seven of the nine diabetic cases and in all cases of the nondiabetic group. Statistically the average maximum decrease rates between the two groups were not significantly different.

Blood sugar. In nondiabetic group, blood sugar decreased from the 1st hour through the 3rd hour, but it elevated abruptly at the 4th hour exceeding the initial value. In diabetic group, blood sugar decreased

TABLE I

Blood 7 min. P, Inorg. P and Blood Sugar after Injection of Glucuronic Acid

Individual Case No.	Age	Sex		Amount of blood chemical components (mg%)				
				Before the injection	Time (hrs) after the injection			
					1	2	3	4
Nondiabetic group								
1	52	M	Blood 7 min. P	3.8	3.7	3.5	4.0	3.7
			Inorg. P	3.7	3.7	3.7	3.6	3.7
			Blood sugar	100	109	114	97	106
2	32	M	Blood 7 min. P	4.6	4.0	4.1	4.3	4.8
			Inorg. P	4.0	3.4	3.9	3.7	3.3
			Blood sugar	118	89	92	120	136
3	21	F	Blood 7 min. P	4.6	4.4	4.7	4.9	4.7
			Inorg. P	4.2	4.0	4.2	4.1	4.1
			Blood sugar	97	95	92	100	111
4	44	M	Blood 7 min. P	4.4	4.2	4.7	4.6	4.6
			Inorg. P	4.7	4.7	4.3	4.6	4.3
			Blood sugar	133	116	118	121	132
5	24	M	Blood 7 min. P	5.2	5.1	5.3	5.1	5.2
			Inorg. P	5.0	4.8	4.8	4.7	4.6
			Blood sugar	109	107	80	87	109
6	22	M	Blood 7 min. P	3.7	3.5	3.8	4.6	4.2
			Inorg. P	4.9	4.9	4.3	4.3	3.9
			Blood sugar	114	104	121	111	123
7	30	F	Blood 7 min. P	3.4	2.6	3.0	2.6	2.7
			Inorg. P	3.2	2.7	3.2	3.1	3.0
			Blood sugar	104	116	127	118	121

Diabetic group

8	66 M	Blood 7 min. P	2.8	2.4	2.9	2.1	2.9
		Inorg. P	2.4	2.2	2.5	2.1	2.3
		Blood sugar	123	118	112	106	106
9	58 F	Blood 7 min. P	3.0	2.6	3.0	2.4	3.0
		Inorg. P	2.8	2.5	2.7	2.5	2.9
		Blood sugar	164	158	151	142	133
10	17 M	Blood 7 min. P	4.0	2.7	3.9	3.7	4.0
		Inorg. P	3.4	3.3	3.7	3.3	3.5
		Blood sugar	165	153	135	120	111
11	24 M	Blood 7 min. P	3.8	2.9	3.9	4.1	4.6
		Inorg. P	3.1	3.0	3.0	3.0	2.8
		Blood sugar	193	182	172	163	167
12	56 M	Blood 7 min. P	3.9	3.3	3.6	3.9	3.9
		Inorg. P	2.7	2.6	2.5	2.2	2.7
		Blood sugar	228	232	204	194	187
13	57 M	Blood 7 min. P	3.3	3.0	3.7	3.3	3.3
		Inorg. P	2.8	3.0	1.9	2.5	2.7
		Blood sugar	123	125	128	109	106
14	63 F	Blood 7 min. P	3.0	3.6	3.6	4.0	3.9
		Inorg. P	2.1	2.3	2.7	2.8	2.8
		Blood sugar	127	120	114	109	106
15	59 F	Blood 7 min. P	5.7	5.1	6.1	6.7	6.3
		Inorg. P	3.3	3.7	3.8	3.5	4.0
		Blood sugar	132	125	119	114	109
16	41 M	Blood 7 min. P	3.3	3.9	3.7	3.8	3.3
		Inorg. P	3.7	3.1	2.5	2.2	2.8
		Blood sugar	189	160	151	147	133

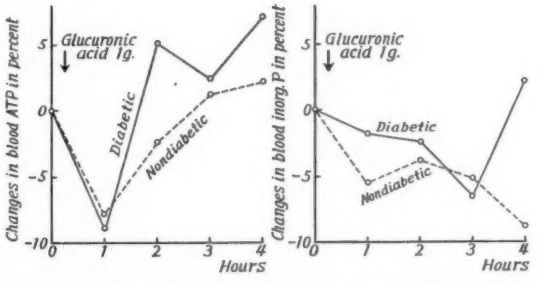

Fig. 1. Changes of blood ATP and inorganic phosphorus caused by the injection of glucuronic acid. The solid lines represent the average values of diabetic patients and the broken lines represent those of nondiabetic subjects.

gradually through the whole test hours in all cases ; however, this decrease seems to be a spontaneous depression of blood sugar due to the daily biorhythm of blood sugar, as observed previously in diabetic patients.[7]

Discussion

It was unmasked recently that glucuronic acid is an intermediate

in uronic acid pathway of carbohydrate metabolism.[14] Our knowledge on the metabolic pathway of glucuronic acid, however, is still far from complete. The fact is found firstly by us and confirmed by other investigators that the diabetic symptoms are improved by the administration of glucuronic acid (sodium glucuronate[6] and glucuronolactone[8]). Konno demonstrated the increase of liver glycogen content after the injection of glucuronic acid in normal rabbits.[9] Shioya and Ohashi found that the administration of glucuronic acid alimeorated the symptoms of diabetes evoked by cortisone in rats.[10] Tazaka and his co-workers showed that glucuronic acid accelerated the oxidation of C^{14}-pyruvate by the in vitro experiment.[11] The increase of blood glucuronic acid was observed by Saltzman and his colleagues in diabetic patients.[12] Similar findings were obtained by Brox. He found that the urinary excretion of glucuronic acid increased in diabetic group and this increase was greater in the cases with diabetic ketosis.[13] We speculate that these increase in glucuronic acid is a reaction to detoxitize the harmful metabolites which are produced during diabetic ketosis and do not degrade through their normal pathways because of the deranged metabolism of diabetes mellitus.

In the present experiment, the injection of glucuronic acid produced an increase in blood ATP and a decrease in blood inorganic phosphorus. These changes are resemble to those produced by the administration of glucose. From the present results, we feel that glucuronic acid accelerates the carbohydrate metabolism in diabetes as well as in nondiabetics. The further studies would be required to clarify the detailed mechanism of this action.

Summary

The effects of glucuronic acid on blood ATP (adenosine triphosphate), inorganic phosphorus and blood sugar were observed in nine diabetic patients and seven nondiabetic adults. The blood ATP (measured as seven minutes' hydrolyzed phosphorus) was elevated distinctly after an initial decrease by the intramuscular injection of 1000 mg. of sodium glucuronate. This injection caused a decrease in blood inorganic phosphorus and also a depression of blood sugar in diabetics as well as in nondiabetics. There were no statistically significant differences in the maximum increase rate (in per cent) of blood ATP, and the maximum decrease rate of blood inorganic phosphorus between the diabetic group and the nondiabetic group.

References

1) Goto & Ito, Tohoku J. Exper. Med., 1957, **67**, 65.
2) Goto, Ito & Ujiie, *ibid.*, 1958, **68**, 51.

3) Goto & Numazawa, *ibid.*, 1958, **68**, 57.
4) Ito, Tohoku Igaku Z. (Jap.), 1957, **56**, 592.
5) Ujiie, *ibid.*, in press.
6) Kurokawa, Goto, Takanami, Ujiie, Seino, Matsushima, Ito & Muraki, Reports on the study of glucuronic acid, The Glucuronic Acid Research Conference, Tokyo, 1958, 5.
7) Goto, Metabolism, 1955, **4**, 323.
8) Nakajima, Ida & Okamura, Rinsho Naikashonika (Jap.), 1957, **12**, 811.
9) Konno, Tohoku Igaku Z. (Jap.), in press.
10) Shioya & Ohashi, Reports on the study of glucuronic acid, 1957, p. 57, Tokyo.
11) Tazaka *et al.*, *ibid.*, 1957, p. 58.
12) Saltzman, Caraway & Beck, Metabolism, 1954, **3**, 11.
13) Brox, Dtsch. Zschr. Verdauungs- u. Stoffwechselkh., 1953, **13**, 193.
14) Horecker and Hiait: New Engl. J. Med., 1958, **258**, 117, 225.

The Tohoku Journal of Experimental Medicine, Vol. 69, Nos. 2-3, 1959

Nucleic Acids

XII. Comparison of DNA from Gastric Cancer with That from Gastric Mucosa

By

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(Received for publication, December 15, 1958)

Sakamoto[1] tried in our Institute in 1957 to conjecture cancerous change in structure of DNA by comparing analytical figures obtained with elaborated methods of human spleen DNA with those of human placenta DNA. Therein, he substituted placental DNA for the cancerous, because placenta resembles cancer, growing rapidly and its components showing intensive skin reaction in cancer patients. On the other hand, he assumed splenic DNA as a typical representative of normal DNAs in a kind of animal, which show some organ-specificity, being based on the findings of Masamune and others including him,[2] although Chargaff[3] had put forward that DNA is generally species-specific but not organ-specific. No noticeable discrepancy was found in composition between the DNA of placenta and that of spleen, whereas the ultraviolet absorption spectra and viscosities pointed to lower polymerization grade of the placental DNA than the splenic. Furthermore, the two DNAs differed in optical rotation and skin reaction in cancer patients. With respect to the components particularly the bases, however, Masamune and others[2] reported before that DNA of Yoshida sarcoma is divergent from those of rat spleen and liver, conflicting with the finding of Sakamoto. These our former co-workers, unlike predecessors who will be mentioned below, thus came to a conclusion affirming cancerous deviation in composition of DNA.

Formerly in 1953, Chargaff & Lipshitz[4] compared DNA from the metastasis[5] portion of carcinomatous human liver with those from the unaffected portion of the same liver and human normal liver, and said that they showed similar compositions. In the same year, Khouvine and Grégoire[6] analyzed DNAs isolated from rat atypical epithelioma, its necrotic part and its metastasis, and stated that they are of almost the same composition, all the DNAs belonging to type AT notwithstanding that they contain less adenine and a little more thymine than DNAs from rat testicles and mar-

row. In 1954, Woodhouse[7] analyzed DNAs from animal (mouse, rat) and human tumors, taking those from calf thymus nuclei, rat liver, human spleen, human foetal thymus and wheat germ as controls. "The ratios of adenine to guanine and thymine to cytosine were greater than 1.0 in all specimens and in this respect the tumor-DNA resembled that from noncancerous animal tissues". The most significant deviation was low thymine value of 0.87 (molar ratio relative to adenine=1.0) of mouse-sarcoma DNA, because the thymine value varied within the limits 0.92 and 1.20 in DNAs from other mouse tumors and rat and human tumors.

Those confusing results suggest that the cancerous change of DNA in composition, if it occurs, does not reach beyond such an extent that can be recognized merely by exact comparison of DNAs of the cancerous tumor and of the corresponding normal tissue, that is, the normal tissue, wherein the cancer originated, with each other, which is the case with respect to neutral glucidamins and mucopolysaccharides.[8]

The writer prepared the DNAs of human gastric cancer and of human gastric mucosa by the mild procedure Sakamoto[1] had preferred and scrupulously examined them chemically by the combined analytical techniques of Masamune and Sakamoto[9], physically and biologically.

Experimental

Preparation of the Deoxyribonucleic Acids as Sodium Salts

Gastric cancers and mucosae of uncancered stomachs, extirpated or teared off, were each washed, having freed from the adherent noncancerous tissue and the necrotic portion in the case of the cancers, masticated and dried in vacuo over H_2SO_4 at room temperature.

Prep. 1 (*mucosa DNA*). 130 g. of 15 dry materials combined was pulverized, suspended in 1.3 *l.* of 0.14 *M* NaCl containing 0.01 *M* sodium citrate (pH 6.8) and stirred in a freezing mixture (0–—10°C) for 24 hours. Centrifuged. The centrifugate was agitated in the same volume of the salt solution as that of the supernatant and centrifuged, followed by two repetitions of the same treatment. The final insoluble residue was extracted with one 300 cc. and three 100 cc. portions of 10% NaCl under chilling as above, each extraction taking 24 hours. The solutions amounting to 500 cc. in total (yellowish brown colored; opalescent; pH 6.6) were poured into 2 volumes of 99% ethanol, chilled to 0°C. The yellowish brown fibrous precipitate that had occurred was taken up in 300 cc. of 10% NaCl in a homogenizer at 0°C, the small insoluble part was removed after centrifugation, and the turbid supernatant was precipitated with ethanol similar to above. Reprecipitation was effected four more times. The nucleoprotein thus given (DNP) was homogenized with 180 cc. of 10% NaCl and deproteinized by effecting Sevag procedure fourteen times,

and the uncolored, transparent solution was poured into 2 volumes of abs. ethanol. The white sodium deoxyribonucleate fibers that had appeared were dissolved in distilled water, precipitated by pouring into 4 volumes of abs. ethanol, and after washing with 3 changes of 66% ethanol, 3 changes of 80% ethanol, 3 changes of abs. ethanol and one portion of ether in succession, dried in a vacuum desiccator containing anhydrous calcium chloride. Yield 170 mg.

Prep. 2 (*mucosa DNA*). 135 g. of 14 dry materials combined was washed five times by stirring in 1.35 *l.* of 0.14 *M* NaCl, containing 0.01 *M* sodium citrate and chilled to 0–5°C, for 48 hours. The tissue residue thus freed from PNA was exhausted three times with 400 cc. of chilled 10% NaCl for 48 hours, and the solutions were subjected to the procedure that had been applied to the corresponding solutions when Prep. 1 was prepared. Yield 165 mg.

Prep. 3 (*mucosa DNA*). Here 70 g. of 7 dry materials combined was taken and washed with 0.7 *l.* of the citrate-containing NaCl solution for 24 hours three times. The following treatments were similar to those when Prep. 1 was prepared. Yield 152 mg.

Prep. 1′ (*cancer DNA*). 40 g. of 8 dry materials combined was agitated in 0.4 *l.* of the citrate-containing NaCl solution once for 48 hours and three times for 24 hours. The insoluble residue was extracted with 300 cc. of 10% NaCl three times, each extraction taking 24 hours. Precipitation of the DNP-solutions and isolation of sodium deoxyribonucleate were processed as in the case of obtaining Prep. 1. Yield 185 mg.

Prep. 2′ (*cancer DNA*). 20 g. of 3 dry materials combined was used. The treatments processed in preparing Prep. 1′ were applied, but 24 hours' washing with the citrate-containing NaCl solution was effected four times instead of three times. Yield 72 mg.

Properties of the Preparations

Test-tube tests. All the preparations gave the negative result in biuret-, Molisch-, Goldschmiedt- and Neuberg-Saneyoshi-test and the almost negative in Bial-test but the markedly positive in Dische diphenylamine test.

The preparations were dried to a constant weight over P_2O_5 in vacuo at 105°C prior to the following examinations.

Quantitative analysis. The analytical figures and the mutual ratios of the bases are shown in Table I.

The cancerous preparations were contaminated with more of PNA than the noncancerous. Of the purines, adenine was less a little and guanine was less to a somewhat larger extent in the cancerous DNA preparations than in the noncancerous as expressed in per cent as well as in molecular ratio. Of the pyrimidines, cytosine was decidedly more in the

TABLE I-A

Composition of the DNAs from Human Gastric Mucosa and Gastric Cancer

Source of DNA	Prep. No.	N* %	P† %	Ash‡	N/P	Diphenylamine reaction of deoxyribose§ $E^{0.05\%}_{1\,cm}(\lambda 595\,m\mu)$	Cysteine reaction of deoxyribose// $E^{0.1\%}_{1\,cm}(\lambda 490\,m\mu)$	PNA¶ %
Gastric mucosa	1	12.98	7.96	23.3	1.63	0.922	0.862	2.2
	2	13.17	7.70	21.3	1.73	0.868	0.814	2.5
	3	13.23	7.72	24.6	1.72	0.875	0.832	3.0
Gastric cancer	1′	13.37	7.79	21.2	1.72	0.840	0.804	4.3
	2′	13.59	7.85	22.8	1.73	0.864	0.820	3.8

* Mikro Kjeldahl. † Plimmer method[10]. ‡ Pregl method without use of H_2SO_4. § Seibert[11]. // Stumpf[12]. ¶ Ribose was estimated by Dische cysteine-H_2SO_4 method[13] and expressed in terms of free yeast PNA E. Merck purified according to Vischer & Chargaff[14].

TABLE I-B

Contents of Bases in the DNAs from Human Gastric Mucosa and Gastric Cancer

Source of DNA	Prep. No.	In per cent				Molecular ratio calcd. to a total of 4.0				Molecular ratio		
		Adenine*	Guanine*	Cytonine*	Thymine*	Adenine	Guanine	Cytosine	Thymine	Adenine Guanine	Thymine Cytosine	Purine Pyrimidine
Gastric mucosa	1	9.1	7.0	4.8	9.4	1.17	0.80	0.75	1.29	1.46	1.71	0.97
	2	9.4	6.8	4.7	9.7	1.21	0.78	0.66	1.35	1.55	2.05	0.99
	3	9.6	6.9	4.4	9.6	1.22	0.80	0.67	1.31	1.53	1.96	1.02
Gastric cancer	1′	9.1	6.5	5.3	9.7	1.15	0.74	0.80	1.31	1.55	1.64	0.90
	2′	9.2	6.3	5.3	9.5	1.17	0.72	0.81	1.30	1.63	1.61	0.90

* Masamune and Sakamoto's technique[9] was applied, and the amounts of the bases were calculated by taking the following values as their molecular extinctions in 0.1 *N* HCl: adenine, 13.0×10^3 (λ 260 mμ)[15]; guanine, 11.0×10^3 (λ 250 mμ)[15]; cytosine, 10.0×10^3 (λ 276 mμ)[16]; thymine, 7.95×10^3 (λ 265 mμ)[15].

cancerous preparations than in the noncancerous, whereas thymine content did not vary from DNA to DNA.

Ultraviolet absorption. 2 mg% solutions of the preparations in a phosphate buffer of pH 7.0 and I 0.147 were examined in a Beckman type

spectrophotometer (EPU-2A type, Hitachi). They absorbed ultraviolet light with maximum between 257 and 259 mμ and minimum between 229–230 mμ. The $\varepsilon(P)_{max}$ values of the cancerous preparations were higher than the corresponding values of the noncancerous. Fig. 1 shows ultraviolet spectra of one preparation each of the cancerous and noncancerous DNAs.

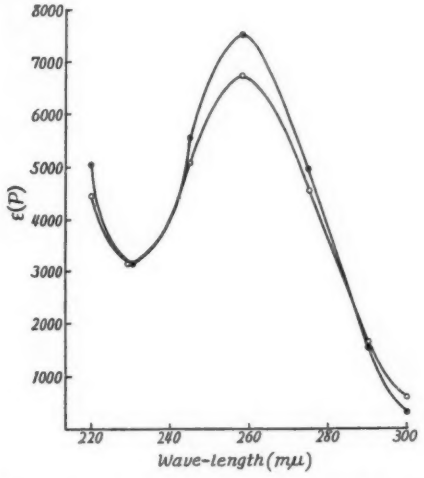

Fig. 1. Absorption spectra of 2 mg% solutions in phosphate buffer, pH 7.0 and I 0.147 of Prep. 1 of stomach mucosa DNA (○—○) and of Prep. 1′ of stomach cancer DNA (●—●).

Optical rotation. 0.3% solutions of the preparations in the same solvent as above gave $[\alpha]_D$ values in Table II, which indicate that the cancerous DNA is much less dextrorotatory than the noncancerous like as the placental DNA is than the splenic.[1)]

Skin reaction. 0.1 cc. volumes of 1/15% (w/v) solutions of Prep. 1′ and 1 were injected intracutaneously into uterus carcinoma patients and the noncancerous female patients, as described in the article of Sakamoto[1)]. The average diameters of rednesses are shown in Fig. 2, from which it is assumed that the cancerous DNA, like placental DNA[1)17)], is provided with the potency of giving more intensive skin reaction in cancer patients than in the noncancerous. The mucosa DNA lacks such a biological property.

Schiff reaction of the DNAs during the course of incubation with 0.02 N HCl. Sakamoto[18)] mentioned that the purines of placental DNA are less stably linked with deoxyribose than those of the splenic DNA, as judged by Schiff reaction of those DNAs during incubation with a very dilute acid solution.

TABLE II

$[\alpha]_D$ Values of the DNAs in Phosphate Buffer of pH 7.0 and I 0.147

The mucosa DNA		The cancer DNA	
Prep. No.	$[\alpha]_D$	Prep. No.	$[\alpha]_D$
1	+147° (t 16°; c 0.3)	1′	+107° (t 16°; c 0.3)
2	+131° (t 16°; c 0.3)	2′	+101° (t 16°; c 0.3)
3	+138° (t 16°; c 0.3)		

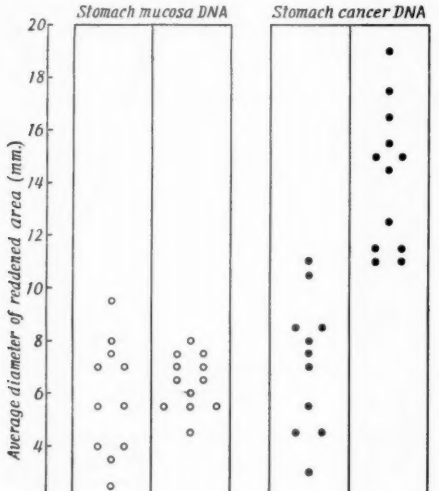

Fig.2. Skin reaction occurring when Prep. 1 of stomach mucosa DNA (○) and Prep. 1′ of stomach cancer DNA (●) were injected to cancerous and non-cancerous patients.

Comparative investigation of the cancer- and mucosa-DNA was extended to the same point by exactly following Sakamoto. The control Schiff analysis was made on "sodium pyrimidine-nucleate", which had been derived from 100 mg. of Prep. 2 according to Sakamoto[19] with a yield of 64 mg. and had a composition summarized in Table III. For most of the reagent samples used,[20] the extinction value (−log T) at 550 mμ after Schiff reaction of that pyrimidine-nucleate increased by 0.115 with 1 mg% concentration increase of its solution analyzed. The main experiment was processed as follows:—40 mg. portions of Prep. 1, 3 and 1′ were

TABLE III

Composition of Sodium Pyrimidine-nucleate Derived from Stomach Mucosa DNA Prep. 2

N* %	P† %	In per cent				Molecular ratio calcd. to a total of 4.0				Diphenylamine reaction[11] $E^{0.05\%}_{1\,cm}$	Schiff reaction[18] $E^{0.004\%}_{1\,cm}$
		Adenine‡	Guanine‡	Cytosine‡	Thymine‡	Adenine	Guanine	Cytosine	Thymine		
5.55	7.57	0.4	0.6	5.3	10.5	0.08	0.12	1.41	2.39	0.860	0.461

* Mikro Kjeldahl. † Plimmer method[10]. ‡ Masamune and Sakamoto[9].

each taken up in 50 cc. of distilled water, an equal volume of 0.02 *N* HCl was added to pH 2.0–2.1, and the mixtures were stoppered and placed in an incubator at 37°C. The intensity of Schiff reaction was determined after Sakamoto at definite intervals during the incubation, taking 2 cc. portions out of the mixtures. The results are illustrated in Table IV and Fig. 3.

The extinction after Schiff reaction of the DNAs arrived at maximum 6 hours after the beginning of incubation, enlarging much more quickly in the cancerous DNA than in the mucosa DNA, and remained constant at least within another 16 hours, suggesting that purines of the cancer DNA are linked less stably with deoxyribose than those of the mucosa DNA. In this respect also, the cancer DNA was to the mucosa DNA what the placental DNA was to the splenic. Moreover, the maximum extinction value amounted more in the cancer DNA than in the mucosa DNA in spite of the smaller total purine content of the former DNA (See Table I). The deoxyribose residues whose 1-position were freed amount more as expressed in per cent of the total deoxyribose, or take the aldehyde form to a larger extent in the cancer DNA than in the mucosa DNA.

SUMMARY

1. DNAs were prepared from human gastric cancer and gastric mucosa according to Sakamoto.

2. Their physical, chemical and biological properties were carefully examined.

3. Adenine amounted less a little and guanine less in a larger grade and cytosine distinctly more in the cancer DNA than in the mucosa DNA.

4. The cancer DNA absorbs ultraviolet light more strongly than the noncancerous.

5. The cancer DNA is less dextrorotatory than the noncancerous.

TABLE IV

Relation of the Incubation Time of the DNAs with Schiff Reaction

Incubating time (hrs.)	Extinction at 550 mμ after Schiff reaction					
	Stomach mucosa DNA			Stomach cancer DNA, Prep. 1′		
	Prep. 1	Prep. 3	Average	Run 1	Run 2	Average
1	0.080	0.082	0.081	0.105	0.111	0.108
2	0.165	0.170	0.168	0.260	0.263	0.262
4	0.352	0.356	0.354	0.431	0.430	0.431
6	0.475	0.480	0.478	0.495	0.494	0.495
8	0.472	0.475	0.474	0.495	0.492	0.494
10	0.479	0.477	0.478	0.494	0.496	0.495
12	0.477	0.477	0.477	0.490	0.490	0.490
14	0.472	0.466	0.469	0.485	0.489	0.487
16	0.464	0.466	0.465	0.486	0.486	0.486
24	0.456	0.460	0.458	0.476	0.474	0.475
36	0.437	0.444	0.441	0.466	0.463	0.465
48	0.424	0.430	0.427	0.458	0.455	0.457

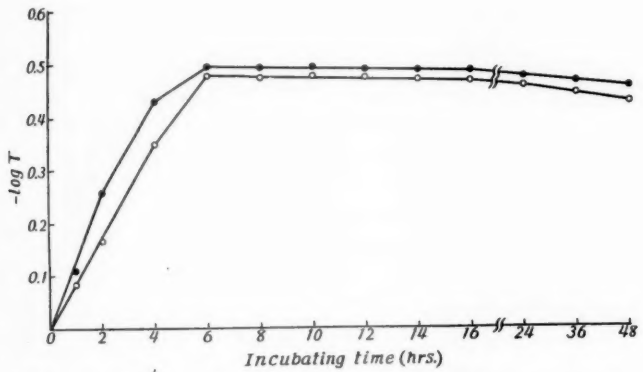

Fig. 3. Showing variation of Schiff reaction with time as expressed in average extinctions at 550 mμ, when 40 mg% solutions in 0.01 *N* HCl of Prep. 1 and 3 of stomach mucosa DNA (○—○) and Prep. 1′ of stomach cancer DNA (●—●) were incubated at 37°C (The average extinction values in Table IV are plotted).

6. The cancer DNA is potent to give stronger skin reaction in cancer patients than in the noncancerous.

7. The cancer DNA shows more intensive Schiff reaction than the noncancerous within 6 hours after the commencement, when incubated

with 0.01 *N* HCl (pH of the solutions 2.0–2.1) at 37°C. The maximum Schiff reaction occurring after the 6th hour is also stronger in the cancerous DNA than in the noncancerous. Namely, the purines are assumed to be bound less stably against acid in the cancerous DNA than in the noncancerous.

In the respects from 5. to 7., the cancer DNA is to the mucosa DNA what the placenta DNA is to the spleen DNA.

The Ministry of Education gave a grant in aid to us, which is gratefully acknowledged. H. Masamune.

References and Notes

1) Sakamoto, Tohoku J. Exp. Med., 1957, **65**, 269.

2) Masamune, Tsuiki, Kitabatake & Sakamoto, *ibid.*, 1955, **61**, 171.

Errata with respect to that article—H. Masamune

i) Page	Line in the text	*Read*	*For*
171	9th from the bottom	The analyses of DNAs of rat-...	The analyses of rat-...
,,	6th from the bottom	But the DNAs of rat thymus...	But the DNAs of thymus...
172	12th from the top	" immature structure " (Masamune) of high-molecular...	" immature structure " in high-molecular...
,,	17th from the top	the rat spleen...	the rabbit spleen...

ii) Read the italicized substitutes for the figures in Table I on p. 175 of the article, which are incorrectly calculated.

TABLE I

Source of DNAs	In per cent	N/P	Molecular ratio calcd. to a total of 4.0				Molecular ratio		
			Adenine	Guanine	Cytosine	Thymine	$\frac{\text{Adenine}}{\text{Guanine}}$	$\frac{\text{Thymine}}{\text{Cytosine}}$	$\frac{\text{Purines}}{\text{Pyrimidines}}$
Rat spleen			*1.16*	*0.84*	*0.75*	*1.25*	*1.38*	*1.67*	*1.00*
" liver									
" thymus			*1.15*	*0.92*		*1.12*	*1.25*	*1.38*	*1.07*
Yoshida sarc.			*1.24*	*0.74*	*0.86*	*1.16*	*1.68*	*1.36*	*0.98*
Pig spleen			*1.30*	*0.77*		*1.11*	*1.69*	*1·35*	*1.07*
Calf thymus									

3) Chargaff, Experientia, 1950, **6**, 201 ; J. Cell & Comp. Physiol., 1951, **38**, 41.

4) Chargaff & Lipshitz, J. Amer. Chem. Soc., 1953, **75**, 3658.

5) The site of the original tumor is not described.

6) Khouvine & Grégoire, Bull. soc. chim. biol., 1953, **35**, 603.

7) Woodhouse, Biochem. J., 1954, **56**, 349.

8) Masamune, Kawasaki, Abe, S., Oyama & Yamaguchi, Tohoku J. Exp. Med., 1958, **68**, 81 ; Kawasaki, *ibid.*, 1958, **68**, 119 ; Masamune, Kawasaki & Abe, S., *ibid.*, 1958, **68**, 173 ; and else.

9) Masamune & Sakamoto, *ibid.*, 1955, **61**, 353.
10) Plimmer, Bioch. J., 1933, **27**, 1810.
11) Seibert, J. Biol. Chem., 1940, **133**, 593.
12) Stumpf, *ibid.*, 1947, **169**, 367.
13) Dische, *ibid.*, 1949, **181**, 379.
14) Vischer & Chargaff, *ibid.*, 1948, **176**, 715.
15) Wyatt, Bioch. J., 1951, **48**, 584.
16) Shugar & Fox, Bioch. et biophys. Acta, 1952, **9**, 199.
17) Masamune, Kanno & Kimura, Tohoku J. Exp. Med., 1954, **60**, 323.
18) Sakamoto, *ibid.*, 1957, **65**, 287.
19) Sakamoto, *ibid.*, 1957, **65**, 281.
20) The experiments were carried out in winter.

The Tohoku Journal of Experimental Medicine, Vol. 69, Nos. 2-3, 1959

Molisch-positive Mucopolysaccharides of Gastric Cancers as Compared with the Corresponding Components of Gastric Mucosae

Fourth Report: On MPSs (Mucopolysaccharides) IV*

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(Received for publication, December 15, 1958)

MPSs IV[1] were prepared from gastric cancers and uncancered gastric mucosae in the manner described already[1] and examined by the methods applied to MPSs I[2] and II[3] with the results below.

Experiments and Comment

Yields of MPSs IV. Group A, AB, B and O cancerous MPSs IV and the corresponding noncancerous MPSs IV were obtained in yields of 0.09, 0.07, 0.06, 0.06, 0.05, 0.04, 0.04 and 0.05% of the respective dry starting materials.

Homogeneity. The preparations proved homogeneous electrophoretically as well as ultracentrifugally[5]. See Fig. 1 and 2. The electrophoretic mobilities suggest that the cancerous substances are a little more acidic than the noncancerous.

Solubility. The eight substances behaved similar in this respect. They are soluble in water adjusted to any pH and 90% (v/v) phenol. Insoluble in the satd. $(NH_4)_2SO_4$ (room temp.), the satd. Na_2SO_4 (33°C), glacial acetic acid, ethanol, acetone and ether. Their solutions in water are precipitated with none of lead acetate, cupric chloride, baryta and trichloroacetic acid.

Test-tube tests. All the substances gave the following result of tests. Molisch ##, Bial, biuret, direct as well as indirect Osaki-Turumi +, Hopkins-Cole (test for tryptophan) ±, Neuberg-Saneyoshi, Dische, tests for phosphorus and hydrolyzable sulfur −.

Paper-chromatography of constituents. It was effected by the procedures

* 226th report of Masamune and co-workers' "Biochemical Studies on Carbohydrates"; 7th report of "Chemical Nature of Toxohormone (Nakahara)".

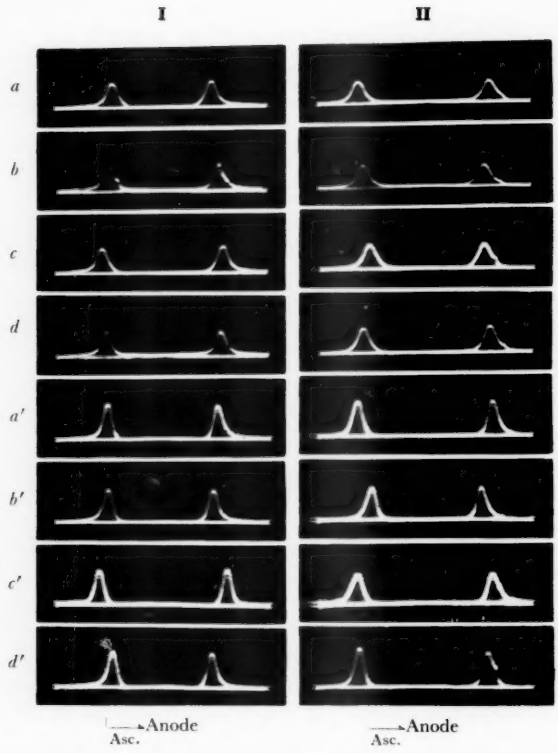

Fig. 1. Electrophoretic patterns of 1% solutions of the cancerous group A(*a*), AB(*b*), B(*c*) and O(*d*) and noncancerous group A(*a'*), AB(*b'*), B(*c'*) and O(*d'*) MPSs IV in veronal-HCl buffer, pH 8.6, I=0.1+NaCl (0.2 *M*) (**I**) after 120 minutes at 2.2 volts/cm and of those in 0.2 *M* acetate buffer, pH 4.6 (I=0.2) (**II**) after 120 minutes at 4.7 volts/cm (temperature 15°C). Examined in a Tiselius apparatus of HTD-1 type, Hitachi. Mobility:

Buffer	Mobility in $cm^2 \cdot volt^{-1} \cdot sec^{-1} \times 10^{-5}$ of the mucopolysaccharides							
	Cancerous MPS IV belonging to				Noncancerous MPS IV belonging to			
	Group A	AB	B	O	Group A	AB	B	O
Veronal-HCl	−2.6	−2.6	−2.5	−2.5	−2.0	−2.1	−2.1	−2.0
Acetate	−0.6	−0.6	−0.6	−0.6	−0.5	−0.5	−0.5	−0.4

similar to those detailed in the Second Report[2] (Hydrolysis agents of the substances: 1 *N* H_2SO_4 for sugar chromatography, and 6 *N* HCl for amino acid chromatography; indicators: 2% aniline phthalate in moist butanol for sugars and 0.2% ninhydrin in moist butanol for amino acids), and any

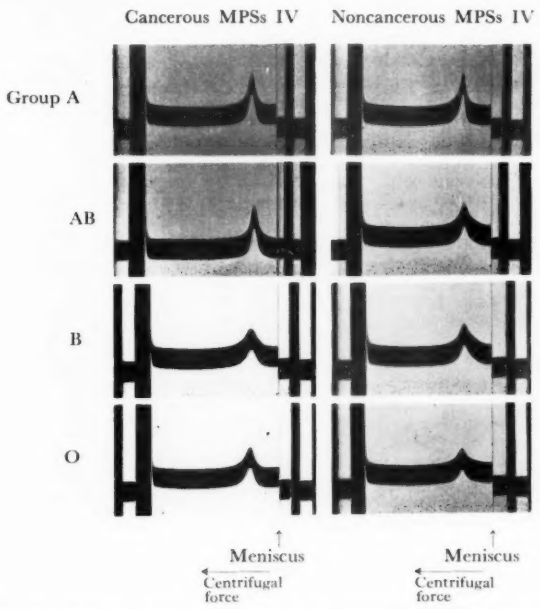

Fig. 2. Sedimentation diagrams of 1% solutions of MPSs IV in *M*/15 phosphate buffer of pH 7.0. The bar angles were 75° excepting that in the case of cancerous group AB MPS IV which was 85°. Conditions else:

Condition	Cancerous MPSs IV				Noncancerous MPSs IV			
	Group A	AB	B	O	Group A	AB	B	O
Mean rotation speed (full speed) (r.p.m.)	59,750	59,800	59,780	59,600	59,950	59,800	59,780	59,750
Mean rotor temperature (°C)	19.6	25.3	30.6	27.4	19.0	23.0	24.5	23.8
Time after reaching full speed (min.)	42	40	40	44	40	40	39	44

of the MPSs IV was found to contain galactosamine, glucosamine, galactose and L-fucose but no glucose nor mannose nor pentoses as sugars, and aspartic and glutamic acids, serine, glycine, lysine, histidine, arginine, threonine, alanine, tyrosine, proline, valine and/or methionine and leucine and/or isoleucine and/or phenylalanine as amino acids. The ninhydrin spots of tyrosine in the chromatograms of cancerous MPSs IV hydrolyzed were very weak in contrast with those in the corresponding chromatograms of the noncancerous MPSs IV which were also not strong. To further identify the possibly overlapping amino acids above, the 6 *N*-HCl hydro-

lysates were also sampled on 60×10 cm. slivers of Toyo Roshi filter paper No. 50 and subjected to descending chromatography with solvent methylethylketone-acetone-water (3:1:0.6 by volume) according to Högström.[4] Temperature 24°±1°C; duration of test 36 hours. The spray reagent was the same that was used to indicate amino acids above. The chromatograms here revealed that the noncancerous substances contain all of valine, methionine, leucine, isoleucine and phenylalanine, but the cancerous the first four only of those five amino acids.

Quantitative composition. The analytical figures are embodied in Table I. Total-hexosamine contents show no marked discrepancies among the

TABLE

Composition of MPSs IV from Stomach

Analysis	In per cent							
	Stomach cancer				Stomach mucosa			
	Group A	AB	B	O	Group A	AB	B	O
N*	4.8	4.8	4.7	4.5	5.5	5.7	5.3	5.4
Total hexosamine†	30.8	33.1	31.7	32.7	32.0	31.9	32.0	30.6
$\frac{\text{Glucosamine}}{\text{Galactosamine}}$ ratio†	1.5	1.7	2.0	1.8	1.4	1.6	1.8	1.6
Galactose‡	28.3	30.1	31.2	30.7	28.1	28.0	30.9	28.0
L-Fucose§	9.0	8.8	9.4	10.3	12.5	11.7	11.6	12.1
Sialic acid//	11.9	10.7	8.1	8.1	3.5	3.8	4.9	4.1
Ash¶	1.5	1.3	1.5	1.4	0.3	0.2	0.2	0.3
Iodine use as glucose**	2.7	3.0	2.7	2.9	4.7	5.4	5.2	5.0

* Micro Kjeldahl. † Masamune and Yosizawa[6]. ‡ Reaction HR_{10} of Masalich and diphenylamine reaction of Werner and Odin[9]; Calcd. as $C_{11}H_{19}NO_9$. ¶

substances belonging to different blood groups nor between the cancerous substances and the noncancerous, being similar to one another. The galactose contents resemble the total-hexosamine contents in the same respects. The noncancerous substances surpass the cancerous in nitrogen- and L-fucose-content. Sialic acid amounts in the cancerous substances even 2–3 times as much as in the noncancerous. The noncancerous MPSs IV consume alkaline iodine distinctly more than the cancerous[11]. As will be described below, the molecular weight of the noncancerous MPS IV belonging to group A is rather higher than the corresponding cancerous mucopolysaccharide. Since iodine consumption depends on the number of reducing terminals, the two findings above look to conflict with each other. Probably, the molecule of the noncancerous substance possesses more branches with an iodine-reducing terminal or terminals per unit

molecule[12] than the cancerous, that is, cancerous deviation is accompanied by less branching in this case. The glucosamine/galactosamine ratio varies from group to group among the cancerous MPSs IV in the same directions as among the corresponding noncancerous, though only to minute extents in the both kinds of MPSs IV. Cf. Kawasaki[2].

Optical rotation. Aqueous solutions of the MPSs IV which were of pH 6.6 were examined. The rotation scarcely differs from group to group in the cancerous substances, while the noncancerous substances are more and more levorotatory in the order from top to bottom of them arranged in the following, the one at the bottom, that is, the noncancerous group O MPS

I

Cancers and Stomach Mucosae

In equivalents per equivalent weight							
Stomach cancer				Stomach mucosa			
Group A	AB	B	O	Group A	AB	B	O
2.18	2.05	1.94	1.88	2.52	2.62	2.20	2.48
1.09	1.11	1.02	1.07	1.14	1.15	1.04	1.10
1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
0.35	0.32	0.33	0.37	0.49	0.46	0.41	0.48
0.25	0.21	0.15	0.15	0.07	0.08	0.09	0.09

mune and Sakamoto[7]. § A modification of Dische and Shettles[8]. // Direct Ehr-Pregl method without use of H_2SO_4. ** Macleod and Robison[10].

IV being most levorotatory.

			$[\alpha]_D^{22}$ (water) (°)
Cancerous MPSs IV	A	substance	$\frac{-0.211\times1}{0.0112\times1}=-18.8$
	AB	"	$\frac{-0.187\times1}{0.0102\times1}=-18.3$
	B	"	$\frac{-0.196\times1}{0.0104\times1}=-18.8$
	O	"	$\frac{-0.224\times1}{0.0109\times1}=-20.6$
Noncanc. MPSs IV	A	substance	$\frac{-0.073\times1}{0.0114\times1}=-6.4$
	AB	"	$\frac{-0.084\times1}{0.0110\times1}=-7.6$

Noncanc. MPSs IV	B	"	$\frac{-0.125\times1}{0.0107\times1}=-11.7$
	O	"	$\frac{-0.174\times1}{0.0108\times1}=-16.1$

All the cancerous MPSs IV are more levorotatory than that noncancerous group O substance.

Ultraviolet absorption. A photoelectric spectrophotometer of EPU-2A type Hitachi was used. Cell 1 cm. thick. Fig. 3 shows the absorption spectra of the cancerous and noncancerous MPSs IV belonging to group A. In general, the cancerous MPSs IV absorbed the ultraviolet light near 270 mμ only faintly, while the noncancerous did distinctly. This coincides with the paper-chromatographic finding that the former substances are devoid of phenylalanine and contain less tyrosine than the latter.

Molecular axial ratio and molecular weight. In the same manner that had been illustrated in the Second Report, following figures were obtained regarding the cancerous and noncancerous group A MPSs IV.

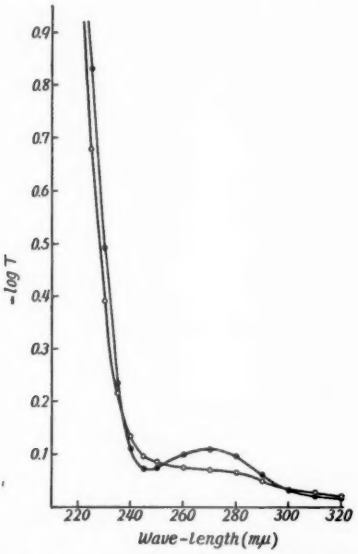

Fig. 3. Ultraviolet absorption spectra of 50 mg% aqueous solutions of pH 6.6 of the cancerous (-○—○-) and noncancerous (-●—●-) group A MPSs IV. Those of other MPSs IV are omitted.

i) Apparent specific volume assumed as partial specific volume V (20°C).

Cancerous A: 0.638
Noncanc. A: 0.636

ii) Volume fraction intrinsic viscosity ν. The intrinsic viscosities $[\eta]$ at 20°C are shown in Table II, wherefrom volume fraction intrinsic visco-

TABLE II

Intrinsic Viscosities of Cancerous and Noncancerous Group A MPSs IV at 20°C

MPS IV		No. of solution	C (Concentration in per cent)	η_r (Relative viscosity)	η_{sp} (Specific viscosity)	η_{sp}/C	$[\eta]$ (Intrinsic viscosity)
Source	Blood group						
Stomach cancer	A	1	0.653	1.655	0.655	1.003	0.770
		2	0.503	1.478	0.478	0.950	
		3	0.387	1.351	0.351	0.907	
		4	0.297	1.259	0.259	0.872	
		5	0.198	1.167	0.167	0.843	
		6	0.165	1.135	0.135	0.818	
		7	0.096	1.078	0.078	0.813	
Stomach mucosa	A	8	0.667	1.543	0.543	0.814	0.625
		9	0.513	1.396	0.396	0.772	
		10	0.394	1.289	0.289	0.734	
		11	0.303	1.216	0.216	0.713	
		12	0.202	1.138	0.138	0.683	
		13	0.168	1.113	0.113	0.674	
		14	0.098	1.063	0.063	0.643	

sities were calculated by equation $\nu = 100\dfrac{[\eta]}{V}$

Cancerous A: 120.7
Noncanc. A: 98.3

iii) Axial ratio of molecule f. The f values were read from curves of f plotted against ν according to Simha.[13]

Cancerous A: 40/1 for rods, 177/1 for disks
Noncanc. A: 35/1 for rods, 144/1 for disks

iv) Sedimentation constant $S_{20,w}$ determined by means of an ultracentrifuge Spinco Model E.[5]

Cancerous A: 5.81×10^{-13}
Noncanc. A: 6.55×10^{-13}

Details are illustrated in Table III.

v) Molecular weight calculated from the axial ratio and sedimentation constant.

Cancerous A: 1.73×10^5 for rods ($f/f_0 = 2.67$)
2.69×10^5 for disks ($f/f_0 = 3.59$)
Noncanc. A: 1.88×10^5 for rods ($f/f_0 = 2.52$)
2.88×10^5 for disks ($f/f_0 = 3.35$)

f/f_0 (frictional ratio) values are calculated according to Perrin

TABLE III

Sedimentation Constants of Cancerous and Noncancerous Group A MPSs IV in $M/15$ Phosphate Buffer, pH 7.0

MPS IV		No. of solution	Concentration (%)	Mean rotation speed (rev/min)	Rotor temperature (mean) (°C)	x Distance of boundary from axis of rotation (cm.)		Δt (sec.)	Δx (x_2-x_1)	Sedimentation constant $\times 10^{13}$	
Source	Blood group					x_1 (initial)*	x_2 (after Δt)			Measured value	$S_{20,w}$
Stomach cancer	A	1	1.00	59,750	19.6	5.988	6.160	1920	0.172	3.97	4.21
		2	0.60	59,950	19.5	5.972	6.176	1916	0.204	4.45	4.73
		3	0.40	59,750	21.1	6.036	6.264	1920	0.228	4.93	5.05
		4	0.20	59,950	22.0	6.016	6.272	1916	0.256	5.53	5.54
Stomach mucosa	A	5	1.00	59,950	19.0	6.036	6.228	1916	0.192	4.15	4.47
		6	0.60	59,800	17.7	6.020	6.240	1920	0.220	4.77	5.31
		7	0.40	59,750	20.2	6.004	6.260	1920	0.256	5.55	5.80
		8	0.20	59,750	21.1	6.044	6.320	1920	0.276	5.94	6.08

* Means x when photographed at first.

formula, neglecting hydration effect.

Blood group potency. The glass plate method Masamune *et al.*[14] had noted was applied, substituting 1% suspensions of erythrocytes for 5% suspensions[15]. The result is illustrated in Table IV. The cancerous A, AB and B MPSs IV are less strongly group A, A and B, or B active than the corresponding noncancerous substances, while there are almost no remarkable differences with respect to group O activity between the corresponding cancerous and noncancerous group substances.

Anemia-inducing potency (*KIK potency*). As to the method used, see Masamune and Kawasaki[16]. As shown in Table V, intravenous injection of even 3 mg. amounts per kg. body weight did not cause anemia in rabbits, that is, MPSs IV proved KIK inactive.

Toxohormone potency. The cancerous and noncancerous group A MPSs IV were examined by employing the titrimetrical and volumetrical methods for catalase determination of Masamune *et al.*[17] The results are seen in Table VI (A–C). The cancerous and noncancerous substances acted as toxohormones when injected intraperitoneally into mice in dosage of 3 mg. and 5 mg. per capita respectively, suggesting that the cancerous MPSs IV are a little more active than the noncancerous.

DISCUSSION

By the investigation renewed, the writers found that the gastric cancer

TABLE IV

Anti-hemagglutinative Potency of MPSs IV of Stomach Cancers and Stomach Mucosae

Goat : a normal anti-O goat serum. Anti-E : an eel serum. Anti-OS : an anti-OS saliva chicken serum absorbed with ABe red cells. − no hemagglutination, ± faint hemaggl., + slight hemaggl., ++ marked hemaggl., +++ strong hemaggl.

MPS IV		Red cells	Serum	Dilution of the substances ($1:10^3\times$)																
Source	Blood group			1	2	2^2	2^3	2^4	2^5	2^6	2^7	2^8	2^9	2^{10}	2^{11}	2^{12}	2^{13}	2^{14}	2^{15}	2^{16}	∞
Stomach cancer	A	A	α	−	−	−	−	−	−	−	−	−	−	−	+	++	+++	+++	+++	+++	+++
		B	β	+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
		O	Goat	−	−	−	−	+	++	++	++	++	++	++	++	++	++	++	++	++	++
		O	Anti-E	−	−	−	+	++	++	++	++	++	++	++	++	++	++	++	++	++	++
		O	Anti-OS	−	−	−	−	−	−	−	++	++	++	++	++	++	++	++	++	++	++
	AB	A	α	−	−	−	−	−	−	−	−	−	±	+	++	+++	+++	+++	+++	+++	+++
		B	β	−	−	−	−	−	−	−	−	−	++	++	+++	+++	+++	+++	+++	+++	+++
		O	Goat	−	−	−	−	++	++	++	++	++	++	++	++	++	++	++	++	++	++
		O	Anti-E	−	−	−	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
		O	Anti-OS	−	−	−	−	−	−	±	+	++	++	++	++	++	++	++	++	++	++
	B	A	α	−	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
		B	β	−	−	−	−	−	−	−	−	−	−	+	++	+++	+++	+++	+++	+++	+++
		O	Goat	−	−	−	−	±	++	++	++	++	++	++	++	++	++	++	++	++	++
		O	Anti-E	−	−	−	−	++	++	++	++	++	++	++	++	++	++	++	++	++	++
		O	Anti-OS	−	−	−	−	−	−	−	−	+	++	++	++	++	++	++	++	++	++
	O	A	α	−	+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
		B	β	+	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
		O	Goat	−	−	−	−	−	−	+	++	++	++	++	++	++	++	++	++	++	++
		O	Anti-E	−	−	−	−	−	−	+	++	++	++	++	++	++	++	++	++	++	++
		O	Anti-OS	−	−	−	−	−	−	−	−	−	−	++	+++	+++	+++	+++	+++	+++	+++
Stomach mucosa	A	A	α	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	++	+++
		B	β	−	−	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
		O	Goat	−	−	−	−	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
		O	Anti-E	−	−	−	−	−	±	+	++	++	++	++	++	++	++	++	++	++	++
		O	Anti-OS	−	−	−	−	−	−	−	+	++	++	++	++	++	++	++	++	++	++
	AB	A	α	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	++	+++
		B	β	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+++	+++	+++
		O	Goat	−	−	−	+	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
		O	Anti-E	−	−	−	−	−	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
		O	Anti-OS	−	−	−	−	−	−	−	+	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	B	A	α	−	−	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
		B	β	−	−	−	−	−	−	−	−	−	−	−	−	−	−	++	+++	+++	+++
		O	Goat	−	−	−	+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
		O	Anti-E	−	−	−	−	+	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
		O	Anti-OS	−	−	−	−	−	−	−	−	++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	O	A	α	−	+	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
		B	β	−	+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
		O	Goat	−	−	−	−	−	−	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
		O	Anti-E	−	−	−	−	−	−	+	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
		O	Anti-OS	−	−	−	−	−	−	−	−	−	−	+	+++	+++	+++	+++	+++	+++	+++

TABLE V

Anemia-inducing Potency of Cancerous and Noncancerous Group A and O MPSs IV

No. of assay	Rabbit			MPS IV injected		Dosis per kg. body wt. (mg.)	Number of red cells ($\times 10^4$, per mm^3)					
	No.	Body wt. (kg.)	sex	Source	Blood group		Before injection	Time after injection (hrs.) 1	2	3	4	5	Maximum decrease (%)
1	15	2.2	♀	Stomach cancer	A	3	674	702	676	685	**634**	655	6
2	16	2.8	♂		A	"	644	665	662	657	674	**642**	0
3	17	3.0	♂		O	"	530	540	539	560	558	553	
4	18	2.0	♀		O	"	642	**630**	644	650	635	647	2
5	19	2.5	♀	Stomach mucosa	A	3	683	672	**666**	680	685	673	2
6	20	2.8	♀		A	"	650	676	655	642	664	**638**	2
7	21	2.5	♂		O	"	593	586	599	580	**574**	590	3
8	22	2.1	♂		O	"	518	510	530	517	**508**	512	2

TABLE VI-A

Liver-catalase Depression *in vivo* by Cancerous and Noncancerous Group A MPSs IV

The average k_{25} values measured regarding each dosis in each test-set are expressed in per cent of the respective corresponding values in the blank controls.

No. of test-set	No. of mice used	Amount of group A MPSs IV injected (mg.)						
		Cancerous IV				Noncancerous IV		
		5	3	2	1	5	3	1
1	9	52.5				75.0		
2	6	59.7						
3	9				80.7			95.4
4	9				79.5			95.1
5	9		61.5		90.4			
6	9		61.3			73.7		
7	6		70.4					
8	6						78.6	
9	6						85.4	
10	6			87.0				
11	6			79.7				
12	6			83.0				
13	6					71.4		

TABLE VI-B

Effect *in vitro* on Liver Catalase of Cancerous and Noncancerous Group A MPSs IV

Liver homogenates were incubated each with 3 mg. of cancerous group A MPS IV or 5 mg. of the noncancerous.

No. of mouse	Cancerous group A MPS IV			Noncancerous group A MPS IV		
	Liver catalase activity (oxygen evolved in cc.)					
	Control I (No incubation	Control II (Incubated without the substance)	Main (Incubated with the substance)	Control I	Control II	Main
119	11.8	9.5	11.0			
120	11.5	9.4	10.0			
121	15.5	13.3	14.5			
122				18.0	16.2	16.9
123				17.9	16.4	17.0
124				14.0	11.7	13.1
Average	12.9	10.7	11.8 (110% of Control II value)	16.6	14.8	15.7 (106% of Control II value)

and gastric mucosa have each four Molisch-positive mucopolysaccharides two of which are group active and the others group inactive. Our former group-mucopolysaccharide preparations[18)-21)] obtained from those tissues were most probably mixtures because they had not been fractioned into individual mucopolysaccharides under the assumption that those tissues contain only one mucopolysaccharide which is group active.

The group active MPSs IV are discriminated from MPSs I, which are also group active, in following points: i) Is surpass IVs in L-fucose content. ii) IVs surpass Is in sialic acid content. iii) The glucosamine/galactosamine ratios of MPSs I are higher than those of MPSs IV. iv) Is are more levorotatory than IVs. v) The electrophoretic mobilities indicate that IVs are somewhat more acidic than Is. vi) IVs have higher axial ratios and heavier molecular weights than Is.

The deviations common to the cancerous group active MPSs I and IV from the corresponding noncancerous MPSs will be enumerated below. Since most of such components of the tumor as mucoproteins and mucopolysaccharides can be isolated as fractions as correspond to those of the corresponding noncancerous tissue, cancerous deviation appears to scarcely change the solubility and precipitability of high molecular tissue components in general.

TABLE VI-C

Effect *in vivo* on Kidney- and Blood-catalase of Cancerous and Noncancerous Group A MPSs IV

3 mg. amounts of canc. group A MPS IV and 5 mg. amounts of the noncancerous were injected intraperitoneally into mice. Liver catalase activities were also determined for comparison.

No. of mouse	Cancerous MPS IV			Noncancerous MPS IV		
	Kidney	Blood	Liver	Kidney	Blood	Liver
$k_{25} \times 10^3$ in blank experiments						
125	7.47	4.98	7.54			
126	5.38	4.00	6.38			
127	6.35	3.76	5.64			
131				4.92	2.81	6.19
132				4.93	4.10	7.06
133				4.00	2.79	8.06
Average	6.40	4.25	6.52	4.62	3.23	7.10
$k_{25} \times 10^3$ in main experiments						
128	5.02	4.41	4.69			
129	7.75	5.16	5.37			
130	5.64	4.25	3.69			
134				6.20	2.32	4.58
135				3.59	3.88	4.90
136				3.20	3.43	5.72
Average (% of the control value)	6.14 (96)	4.27 (100)	4.58 (70)	4.33 (94)	3.21 (99)	5.07 (71)

1) Group potency. Cancerous A and B substances are less A and B active respectively than the corresponding noncancerous. With respect to group O activity, however, not only those cancerous A and B substances but also the cancerous group O substances are nearly equally strong as the corresponding noncancerous group substances are.

2) Sialic acid content. The cancerous MPSs I and IV contain more sialic acid than the corresponding noncancerous.

3) Glucosamine/galactosamine ratio. In the noncancerous group A, B and O MPSs I, the ratio amounts to 2.3, 3.5 and 3.1 respectively, that in the B substance being the highest, as is generally the case among the corresponding group mucopolysaccharides belonging to different groups from the same source.[22] This variation of the ratio according to blood group is no more recognized in the cancerous MPSs I. The noncancerous MPSs IV are almost not given such a group characteristic, the

cancerous MPSs IV much less.

4) Optical rotation. The noncancerous MPSs I as well as IV optically diverge from group to group, whereas the cancerous MPSs I and IV do not. The cancerous mucopolysaccharides are more levorotatory than the corresponding noncancerous.

5) Molecular axial ratio and molecular weight. The cancerous group A mucopolysaccharides are of higher axial ratios and have a little lower molecular weights than the corresponding noncancerous group A substances.

All the cancerous deviations are ascribed to structural change, needless to say.

Summary

1. The MPSs IV denominated in the First Report were prepared as formerly described and examined.

2. Cancerous deviations common to cancerous MPSs I and IV are pointed out.

Through the Grant Committee for Scientific Researches the Ministry of Education gave a grant in aid to us, which is gratefully acknowledged. H. Masamune.

References and Notes

1) Masamune, Kawasaki, Abe, S., Oyama & Yamaguchi, Tohoku J. Exp. Med., 1958, **68**, 81.

2) Kawasaki, *ibid.*, 1958, **68**, 119.

3) Masamune, Kawasaki & Abe, S., *ibid.*, 1958, **68**, 173.

4) Högström, Acta chem. Scand., 1957, **11**, 743.

By the same procedure, the spot corresponding to valine and methionine and that corresponding to leucine, isoleucine and phenylalanine in the chromatograms of MPSs I, hydrolyzed with 6 *N* HCl, were disclosed to have been given by valine and methionine and by leucine and isoleucine respectively. MPSs I do not contain phenylalanine.

5) The writer is indebted to Mr. Y. Amano and Miss R. Ota in the Bacteriological Department for the ultracentrifugal examination.

6) Masamune & Yosizawa, Tohoku J. Exp. Med., 1957, **65**, 169.

7) Masamune & Sakamoto, *ibid.*, 1956, **63**, 345.

8) Masamune & Kawasaki, *ibid.*, 1956, **63**, 369 (ff. 381–382).

9) Werner & Odin, Acta soc. med. Upsal., 1952, **57**, 230.

10) Macleod & Robison, Bioch. J., 1929, **23**, 517.

11) There is no marked discrepancy of iodine use between the cancerous MPSs I and the noncancerous.

12) Cf. Masamune, Proceedings of the International Genetics Symposia, 1956, 593, and else.

13) Simha, J. Phys. Chem., 1940, **44**, 25.

14) Masamune, Hakomori, Numabe, Akama & Kamiyama, Tohoku J. Exp. Med., 1955, **61**, 283 (ff. 294–295).

15) This was also the case when MPSs I and II were assayed, although the writers did not mention it by mistake in the second and third reports (2) and 3)).

16) Masamune & Kawasaki, Tohoku J. Exp. Med., 1956, **63**, 369 (f. 372).

17) Masamune, Tsuiki, Kamiyama, Abe, S., Haga, Kawasaki, Kaketa & Abe, Sh., *ibid.*, 1958, **67**, 309.

18) Satoh, *ibid.*, 1949, **51**, 275.

19) Oh-Uti, *ibid.*, 1949, **51**, 297.

20) Masamune, Yosizawa, Oh-Uti, Matuda & Masukawa, *ibid.*, 1952, **56**, 37.

21) Masamune, Yosizawa & Masukawa, *ibid.*, 1953, **58**, 381.

22) Cf. Kabat, Blood group substances, Academic Press, New York, 1956, p. 151 ; Masamune, H., Hakomori, Masamune, O. & Takase, Tohoku J. Exp. Med., 1956, **64**, 67 ; Masamune, Yosizawa & Tokita, *ibid.*, 1957, **65**, 187.

The Tohoku Journal of Experimental Medicine, Vol. 69, Nos. 2-3, 1959

Biochemical Studies on Carbohydrates

CCXXVII. On Paper Chromatography of Hexuronic Acids

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There were three papers published from this laboratory with respect to paper chromatography of glucuronic, galacturonic and mannuronic acids.[1)-3)] Since then, L-guluronic acid was isolated as L-guluronolactone from alginic acid,[4)] and L-iduronic acid in β-heparine and skin-itinsulfate was identified by paper chromatography[5)] as well as by isolation as 2,3,4-tri-O-acetyl-L-idosan.[6)] Therefore the writers have investigated how to discriminate those five hexuronic acids occurring in nature in a paper chromatogram, with the resulting conclusions: 1) Kinds of filter paper made up of acid-treated fibers, for instance, Toyo Roshi filter paper No. 3 give concentrated spots contrary to those of filter paper without previous acid-treatment. 2) Free hexuronic acids are less sharply separated than their lactones by any of the solvent mixtures listed in Table I. 3) Solvent VI, VIII and IX in Table I are most appropriate for the purpose, because they separate galacturonic acid from the remaining hexuronic acids on one hand and the lactones of the latter four hexuronic acids from one another on the other. R_r (rate of flow of a sugar against rhamnose) values of the hexuronic acids and their lactones are shown in the same table. 4) It is advisable to preliminarily free a hexuronic acid or acids from hexosamines, amino acids and ordinary sugars, which are present in the same hydrolysate, by the aid of a cation- and an anion-exchanger, if identification of them in mucoproteins is aimed at by paper chromatography.

Experimental

Hexuronic Acids as Materials

D-Glucuronic acid. Crystalline glucuronolactone (Koso Chemical Co.) was taken up in water, NaOH was added to neutral from time to time to open the lactone ring, and thereafter sodium ions were eliminated by passing the solution through a column of Dowex 50 (H form), followed by dilution of the effluent to 1% with respect to the free hexuronic acid.

D-Galacturonic acid. Crude barium galacturonate (amorphous) pre-

pared from beet pectin was freed from barium by the aid of an Amberlite IR-120 (H form) column, and after again adsorbing on a column of Amberlite IR-410 (acetate form), eluted by means of 0.1 *N* sulfuric acid. The eluate was collected in fractions. The Goldschmiedt positive fractions were united, the sulfuric acid was precipitated with excessive baryta, and the centrifuged supernatant was treated with the cation exchanger above and distilled in vacuo, whereupon a crystalline porridge (needles) was given. The product melted at 107–108°C and browned at about 153°C on further elevation of temperature. $[\alpha]_D^{\circ}$ $(H_2O)=\dfrac{+2.05\times 100}{1.99\times 1}=+103.0°$ (initial, extrapolated)$\longrightarrow\dfrac{+1.06\times 100}{1.99\times 1}=+53.3°$ (at equilibrium). A 1% solution of it was prepared for the paper-chromatographic study.

D-Mannuronic acid. A weighed amount of barium mannuronate (amorphous; a gift from Prof. K. Aso in the Department of Agricultural Chemistry) was taken up in a volume of water, liberated from barium ions with Dowex 50 (H form) and diluted to 1% with respect to the hexuronic acid as free acid.

L-Iduronic acid. 10 mg. of 1,2-O-isopropylidene-L-iduronolactone (a gift from Prof. M. L. Wolfrom, Ohio) was suspended in 1 cc. of 20% acetic acid and refluxed for 160 minutes in a boiling water-bath. The hydrolysate was distilled in vacuo at a temperature below 30°C until it smelled no more of acetic acid. The still-residue was dissolved in 0.4 cc. of water, 0.01 *N* NaOH was added several times, each addition being stopped when the solution became neutral, and the sodium ions were removed by the aid of Dowex 50 (H form). The free hexuronic acid solution thus given was condensed as above to about 1%.

L-Guluronic acid. a) Preparation of sodium L-guluronate. 1.8 g. of 3,6-D-glucosaccharolactone (F.P. 132–133°C) prepared according to Kiliani[7] was taken up in 20 cc. of water and transferred to a three-necked flask. The solution was chilled to 0–1°C, and 60 g. of 2.5% sodium amalgam was added in small portions extending over 30 minutes under vigorous agitation, during which the temperature of the reaction mixture was maintained beween 3–6°C and the pH value of it between 3–4 by adding 20% sulfuric acid dropwise. After stirring for another 20 minutes under continuous chilling, the reaction mixture was filtered, and 10% barium acetate was added in small portions to the filtrate, until no more precipitate occurred. The centrifugate ($BaSO_4$) was washed with changes of warm water, and the supernatant and washings (100 cc. in total) were passed through a 1.2×25 cm. column of Amberlite IR-120 (H form) to remove cations and washed. The effluent and washing were concentrated to a syrup, diluted with 30 cc. of water and neutralized with the saturated

baryta. The centrifuged supernatant and the washing of the centrifugate were combined (63 cc., pH 6.8), added to a 2.6×19 cm. column of Dowex 1 (acetate form), and after washing with 100 cc. of water, eluted with 0.3 *N* acetic acid at a flow rate of 2 cc. per minute. The eluate was collected in 50 cc. fractions. Frs. Nos. 10–19 which were Goldschmiedt positive were distilled together in vacuo at a temperature below 35°C to a thick syrup. It was then dissolved in 5 cc. of water, neutralized carefully with 1.0 and 0.1 *N* solutions of NaOH to a constant pH value of 7.2 and distilled as above to about 1 cc. The condensate containing some crystals in needles was stood overnight in a refrigerator, and the crystalline porridge was filtered off under chilling and washed with dilute methanol and abs. methanol in succession. Yield 570 mg. F.P. 124–125°C (gas evolution). Recrystallized twice from water, it melted constant at 125.5–126°C (gas evolution). $[\alpha]_D^{\circ}(H_2O)=\frac{+0.49\times 100}{1.11\times 1}=+44.1^{\circ}$ (c 1.1). No mutarotation took place. Found: C, 27.22; H, 5.18; ash (Na_2SO_4) (Pregl), 27.33; crystal water, 9.92 (loss at 105°C in vacuo over P_2O_5 for 5 hours). $C_6H_9O_7Na\cdot 2\frac{1}{2}H_2O$ requires C, 27.59; H, 5.40; ash 27.20; crystal water, 17.25. C and H were kindly analyzed by Mr. H. Obara Assistant in the Chemical Department according to Pregl, for which the writers are indebted.

b) Free guluronic acid. A solution of sodium guluronate was freed from sodium by the aid of Dowex 50 (H form) and diluted to 1% with respect to the free acid.

c) L-Guluronolactone. L-Guluronic acid does not readily change into lactone when stored as a solution (examined by paper chromatography). Therefore a part of the 1% solution of L-guluronic acid was evaporated to dryness on a boiling water-bath and further placed in a vacuum-desiccator containing anhydrous calcium chloride for 2 days. The substance thus given was dissolved in water and diluted to 1%.

Method of Paper Chromatography

(7–10.5)×56 cm. slivers of Toyo Roshi filter paper No. 3 were each spotted at a distance of 10 cm. from the upper edge with a volume, containing 200–300 γ in total of a hexuronic acid and its lactone as hexuronic acid, of one of the above solutions, which had been stored in a refrigerator for several days after preparation. Each of the slivers was also spotted in a similar manner with a 200 γ amount of rhamnose Takeda side by side with the spot above, and each spot covered an area about 0.8 cm. in diameter.

Next the rectangular trough in the same apparatus as the one illustrated by Masamune and Yosizawa[3] was filled with one of the solvent mixtures

in Table I, which had been stored for more than 2 weeks at room temperature, and after covering with the bell (The spotted paper-slivers were not hung on the trough), placed in an air thermostat at 24–27°C for 12 hours until the atmosphere of the chamber was saturated regarding the solvent. Then the apparatus was opened and quickly set up, dipping the upper edge of the slivers in the solvent and shutting with the bell. The chromatograms developed in a descending way. Temperature of test the same as above. Duration of test 5–15 hours. Indicator aniline hydrogen phthalate reagent of Partridge[8] or *p*-anisidine phosphate reagent of Mukherjee and Srivastava.[9]

Result

In Table I are embodied average R_r values of hexuronic acids and their lactones by various solvents, which had been calculated after 3–8 runs of chromatography with each solvent regarding each hexuronic acid. Fig. 1 is plotted from those figures.

Discussion and Remarks

1) Fischer and Dörfel[10] disapproved our previous reports[2),3)], saying that spots of hexuronic acids tail and diffuse by the solvents in those reports. Such an inconvenience occurs as a matter of fact, when such papers as Toyo Roshi filter paper Nos. 2, 50 and 51 which are untreated with acid are used, but does not when the acid-treated Toyo Roshi filter paper No. 3 and the alike are used, as is generally the case in paper chromatography of organic acids.[11]

2) Masamune and Yosizawa[3] obtained R_r values of D-mannuronolactone and D-glucuronolactone in reversed relationship with those of the same lactones in this account. It is probably because the mannuronic acid of Masamune and Yosizawa was not pure, giving more than two spots, whereas the corresponding material here gave only two spots one spot of free mannuronic acid and one spot of mannuronolactone, and in addition those authors applied the acid-untreated Toyo Roshi filter paper No. 2.

3) Preliminary treatment of the acid-hydrolysates with a cation-exchanger or with a cation- and an anion-exchanger is requisite for paper chromatography of a hexuronic acid or acids in Goldschmiedt-positive mucopolysaccharides and mucoproteins, as is exemplified in the article of Satake.[12]

4) As was remarked by Masamune and Maki[2], R_f values and even R_r values of sugars are affected easily by many factors, so that it is advisable to run reference hexuronic acids in parallel with the unknown hexuronic

TABLE I

Average R_r Values of Hexuronic Acids by Various Solvents at 24–27°C

No.	Solvent: Composition by volume	Duration of test (hrs.)	Average distance run by rhamnose (cm.)	Average R_r value: D-Glucuronic acid Free	D-Glucuronic acid Lactone	D-Galacturonic acid	D-Mannuronic acid Free	D-Mannuronic acid Lactone	L-Iduronic acid Free	L-Iduronic acid Lactone	L-Guluronic acid Free	L-Guluronic acid Lactone
I[1]	*n*-Butylacetate 5 Pyridine 3 Water 1	5	14.1	0	1.61	0	0	1.29	0	2.36	0	2.30
II[2]	Benzylalcohol 3 Acetic acid 3 Water 1	13	27.6	0.51	0.90	0.49	0.59	0.91	0.66	0.96	0.57	0.98
III[3]	Butanol 5 Pyridine 3 Water 2	10	28.6	0.20	1.08	0.18	0.27	0.94	0.34	1.36	0.21	1.27
IV[3]	*iso*-Amylalcohol 7 Pyridine 7 Water 6	13	26.8	0.30	1.12	0.28	0.38	1.00	0.45	1.44	0.30	1.31
V[3]	*n*-Butylacetate 3 Acetic acid 2 Water 1	10	20.2	0.40	0.95	0.43	0.46	0.87	0.59	1.21	0.44	1.14
VI[3]	*n*-Butylacetate 3 Acetic acid 2 Ethanol 1 Water 1	10	24.6	0.38	0.97	0.33	0.44	0.89	0.61	1.23	0.44	1.12
VII[3]	*n*-Butylacetate 3 Acetic acid 2 Methanol 1 Water 1	7.5	20.1	0.43	0.97	0.44	0.50	0.88	0.68	1.21	0.50	1.18
VIII[3]	*n*-Butylacetate 3 Acetic acid 2 Butanol 1 Methanol 1 Water 1	12	28.0	0.42	0.93	0.38	0.48	0.80	0.67	1.28	0.42	1.06
IX[3]	*n*-Butylacetate 3 Acetic acid 2 Butanol 2 Methanol 1 Water 1	15	28.0	0.38	1.00	0.32	0.42	0.78	0.62	1.32	0.41	1.15
X	Butanol 5 Methanol 2 Water 1	7	21.8	0.38	0.92	0.37	0.46	0.68	0.64	1.24	0.43	1.08
XI	Butanol 5 Methanol 2 Pyridine 1 Water 1	12.5	25.7	0.14	0.95	0.10	0.16	0.76	0.22	1.30	0.13	1.16

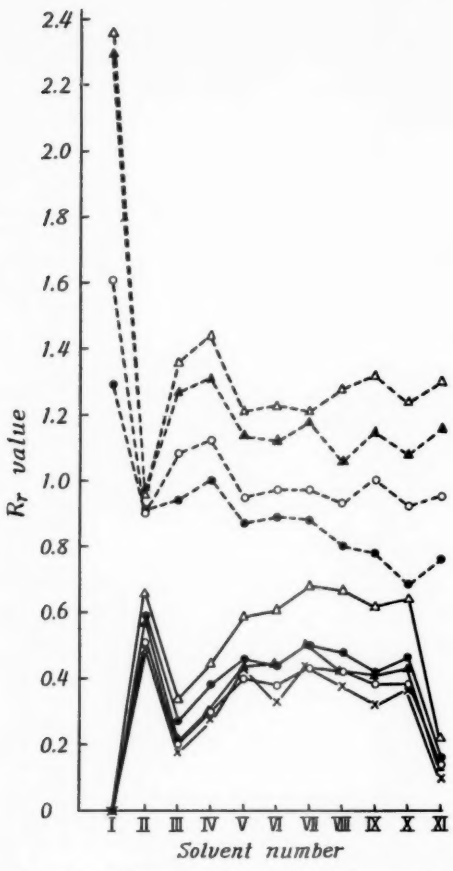

Fig. 1. R_f values of hexuronic acids and their lactones in Table I are plotted against solvent numbers. ○——○ glucuronic acid ; ○- - -○ glucuronolactone ; ×——× galacturonic acid ; ●——● mannuronic acid ; ●- - -● mannuronolactone ; △——△ iduronic acid ; △- - -△ iduronolactone ; ▲——▲ guluronic acid ; ▲- - -▲ guluronolactone.

acid or acids on one and the same paper-strip.

Summary

1. It is described how to discriminate hexuronic acids by paper chromatography.

2. Sodium L-guluronate was prepared and examined.

The Ministry of Education gave a grant through the Grant Committee for

Medical, Dental and Pharmaceutical Researches to defray the expenses of this work, which is gratefully acknowledged. H. Masamune.

References

1) Yosizawa, Tohoku J. Exp. Med., 1950, **53**, 125.
2) Masamune & Maki, *ibid.*, 1952, **55**, 299.
3) Masamune & Yosizawa, *ibid.*, 1953, **59**, 1.
4) Fischer & Dörfel, Hoppe-Seyler's Z., 1955, **302**, 186.
5) Hoffman, Linker & Meyer, Science, 1956, **124**, 1252.
6) Jeanloz & Stoffyn, Fed. Proc., 1958, **17**, 249.
7) Kiliani, Ber. dtsch. chem. Ges., 1925, **58**, 2344.
8) Partridge, Nature, 1949, **164**, 443.
9) Mukherjee & Srivastava, *ibid.*, 1952, **169**, 330.
10) Fischer & Dörfel, Hoppe-Seyler's Z., 1955, **301**, 224.
11) Block, Durrum & Zweig, A manual of paper chromatography and paper electrophoresis, Academic Press Inc., Publishers, New York, 1955, p. 33.
12) Satake, Tohoku J. Exp. Med., 1958, **68**, 385.

The Tohoku Journal of Experimental Medicine, Vol. 69, Nos. 2-3, 1959

Electromyographic Studies on Urinary Tract

I. Fundamental Studies on EMG of Animal and Human Ureters

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Introduction

Since the investigation by Orbeli and Brücke[1] on the action potential of the ureter many works have been reported by Hasama[2], Aiba[3], Bozler[4], Yano[5], Niu[6][7], Obama[8], Kudo[9] and others, but all these investigations refer to animal ureters, and there are as yet many points of contention concerning the wave patterns observed in these studies. Recently more advanced research methods have become available, so that the results obtained by Sleator[10], Butcher[11] and others, using these advanced methods are more reliable, and thus the research on this subject has come to turn a new page.

The present authors have been interested in this subject and made some observations on the human ureters in comparison with animal ureters; in the present investigation patients with apparently normal urinary organs subjected to laparotomy for intestinal diseases were chosen as subjects, and action potentials of their exposed ureters were recorded over a sufficiently long period. The nature of the wave patterns obtained has been critically studied. The result will be reported in the following.

Experimental

Methods of Observation and Experiment

1) As experimental animals, 42 adult dogs (6–13 kg.) were used. They were fixed in dorsal position in a laboratory 10–25°C in temperature and laparotomized under anesthesia with thiopental-sodium administerated

intravenously in a dosis of 10 mg/kg. Then the retroperitoneum was opened, the ureters were bluntly, detached for 2–4 cm. upwards, from a point about 4–8 cm. above their attachment to the bladder, and action potentials were led off from these exposed parts of the ureters.

2) The human subjects were 12 patients of intestinal diseases clinically diagnosed as having intact urinary organs. After laparotomy their ureters were detached from the retroperitoneum to a length of about 4 cm., in a region somewhat proximal to the line a terminalis, and action potentials were recorded from these exposed parts.

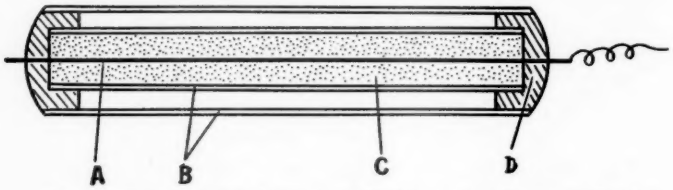

Fig. 1. A is a silver or iron wire 0.05–0.1 mm. in diameter. B, glass pipettes, these cover A. C, (paraffin) D, (sealing wax)

For recording action potentials a four stage amplifier (one CR-coupled and three direct) by San-ei & Co. or the Poly-Viso by Sanborn & Co. was used. A silver wire 0.05–0.1 mm. in diameter covered with a glass pipette except at the tip and fixed with paraffin, served as a different electrode. This electrode was made following a suggestion from Dr. Niu, Kyoto University (See Fig. 1). In some cases an iron wire of the same diameter was used instead of the silver wire. The electrode was introduced into the muscle layer of the ureter through its adventitia and kept suspended so that it was movable in accordance with the peristaltic movement of the ureter.

For unipolar leading, the different electrode was placed in the ureter, and the indifferent electrode of similar construction in the fatty tissue around the ureter. For bipolar leading one of the electrodes was placed at the proximal and the other at the distal end of the exposed ureter.

The recording instrument used was, the heat-writing recorder attached to the Poly-Viso or the H-type vibrator of Yokokawa's electromagnetic oscillograph. In records, the full length of a vertical bar represents 1 mV and that of a horizontal bar 1 or 10 seconds.

Experimental Results

Wave patterns. As shown in Fig. 2A (dog) and 2B (man), four-phasic action potentials appeared one after another with certain regular intervals corresponding with peristalsis. Action potentials of man and dog are quite similar in wave-form. Upon recording these waves at a

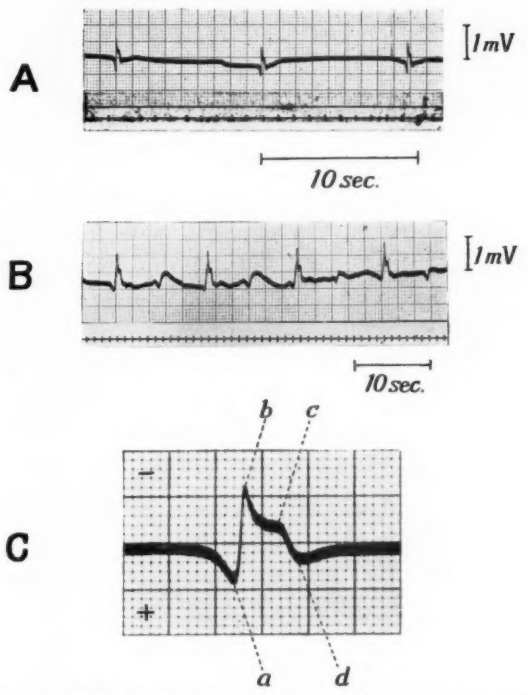

Fig. 2. A shows EUG in normal dogs. B shows EUG in human subjects. C is a typical wave pattern in dog or man. *a*, positive preceding deflection. *b*, major negative deflection. *c*, minor negative deflection. *d*, positive after potential.

higher velocity, we found, as shown in Fig. 2C, that a conspicuous major negative deflection and an accompanying minor negative deflection came between a positive preceding deflection and another positive after-potential.

Besides such a typical wave pattern, some cases, human or canine, were found to show potentials in which the minor negative deflection, and both positive deflections were less prominent. The wave patterns could be classified into the 3 following types (Figs. 3 and 4). Besides the typical wave pattern mentioned above (Type I), we found cases with indistinct minor negative deflection (Type II) and cases in which the principal negative deflection alone was remarkable, but both positive deflections were lacking (Type III). Fig. 3 shows potentials recorded with the silver electrodes, the amplifier having a time constant of 10 sec. and the Yokokawa's oscillograph while Fig. 4 shows those recorded with the iron electrodes, Poly-Viso having a time constant of 2.5 sec. and the heat-writing

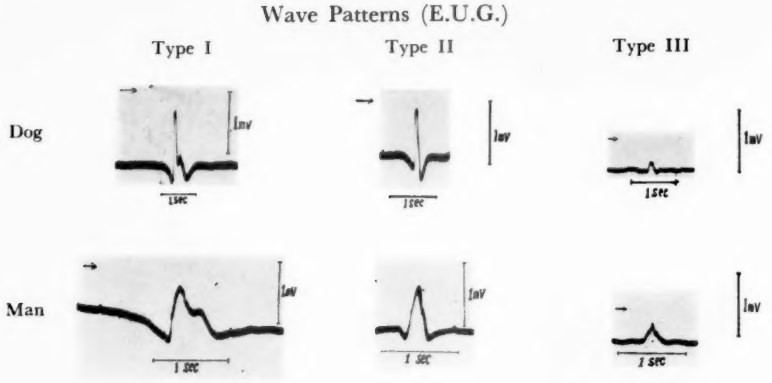

Fig. 3. For recording action potentials a four stage amplifier (one CR-coupled and three direct, time constant : 10 sec.) by San-ei & Co., the silver electrodes and the Yokokawa's oscillograph were used. Type I, typical wave pattern ; Type II, with indistinct minor negative deflection ; Type III, principal negative deflection alone. The arrow-marks show the direction of progress.

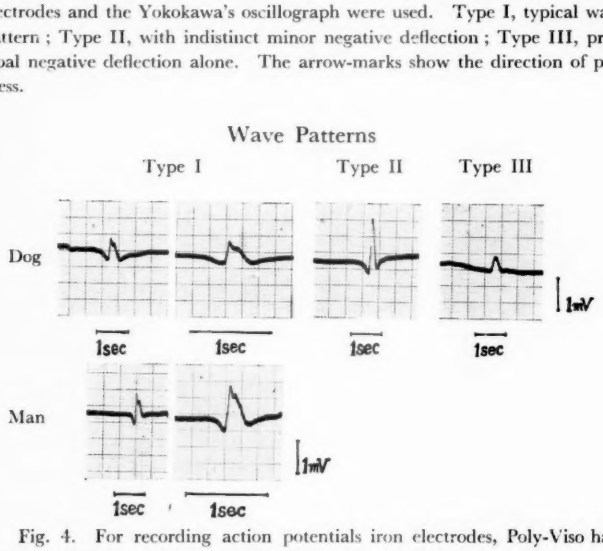

Fig. 4. For recording action potentials iron electrodes, Poly-Viso having time constant of 2.5 sec. and heat-writing recorder were used.

recorder. Some minute differences are found between the wave-patterns shown in Fig. 3 and Fig. 4, but these differences cannot be deemed essentially significant.

The frequency of appearance of the three types classified above was as follows. Among the 42 dogs tested, Type I occured in 35 tests, Type II in 28 tests and Type III in 17 tests, while among the 12 human subjects, Type I

TABLE I
Frequency of Appearance of the Three Types

	No. of cases	Type I	Type II	Type III
Dogs	42	35	28	17
Men	12	8	8	2

came forth in 8 tests, Type II also in 8 tests and Type III in only 2 tests. The frequency of Type III was thus low in dog as well as in man (Table I).

Such wave patterns are stable, for the same wave pattern would persist for 1–3 hours or even longer, as far as the electrical contact between the electrodes and the ureter remains unchanged. When, however, these conditions were changed, we found in many cases that the wave pattern changed over to another type.

When the uretral peristalsis was powerful, Type I appeared most frequently, Type II coming next and Type III being least frequenty, but when the peristaltic movement was weak, Type III was encountered most frequently, Types I and II being far less frequent. Besides, we encountered Type III very often when the room temperature fell below 10°C, but Types I and II came forth more frequently when the temperature stood between 17° and 25°C.

Discharge intervals. The intervals of the electric discharges showed individual differences and also varied with the lapse of time in the same individual as shown in Fig. 5, we found 3 general types here too, namely, the cases with an approximately constant interval between the successive discharges, the cases showing cyclic changes consisting of two or three regualar discharges followed by a pause and the cases where the discharges occurred suite irregularly. Such interval patterns, however, were not constant per cases, for the discharges tended to occur at regular intervals at the initial stage of recording but tended to become interrupted by pauses or irregular in appearance with the lapse of time in many cases. The intervals tended to be equal and regular when the peristaltic movement of the ureter seemed strong, but irregular when only weak peristalsis was observed.

In the human subjects tested, the patterns of discharge intervals were nearly similar to those in the experimental animals, but irregularity in the intervals was more frequent among the former.

Measurements of discharge intervals, amplitude and duration. As shown in Fig. 6, a discharge interval was measured by the number of seconds elapsed between two consecutive positive preceding deflections, *a* and *a'* the amplitude H (mV) by the vertical distance from *a* to the apex of the major negative discharge, *b*, and the duration F (sec.) of the potential by the

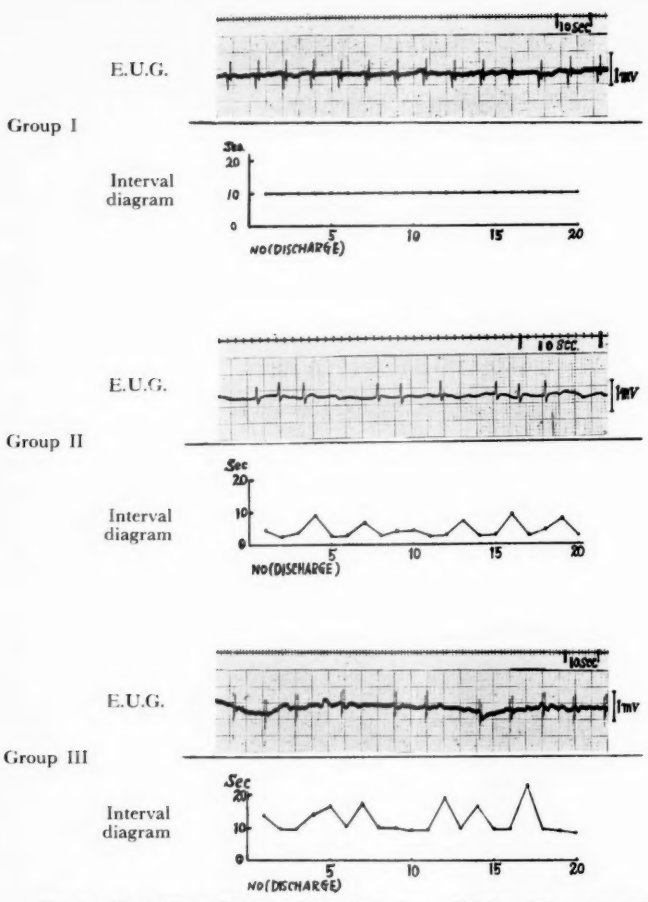

Fig. 5. Variation of discharge intervals in dogs. We found three general types. Group I, regular and constant intervals. Group II is showing cyclic change consisting of two or three discharges followed by a pause. Group III is showing quite irregular intervals.

time from *a* to the apex of the positive after-potential, *d*.

The measured values of the discharge intervals, including the irregular intervals described above, ranged between 1 and 20 seconds in dogs and between 5 and 40 seconds in human subjects, the dogs in general showing shorter intervals (Fig. 7).

The amplitude of the discharges ranged between 0.5 and 1.5 mV in dogs and between 0.3–1.7 mV in men, the human and the canine subjects

showing very small difference in this respect. The amplitude did not show any tendency to rise or fall with the lapse of time, in any test with the same ureter under the same test conditions (Fig. 7).

Fig. 6. Measurements of discharge interval, amplitude and duration. *a*, positive preceding deflection ; *b*, major negative deflection ; *c*, minor negative deflection ; *d*, positive after potential. *aa'*, discharge interval (sec.) ; H, amplitude (mV) ; F, duration (sec.).

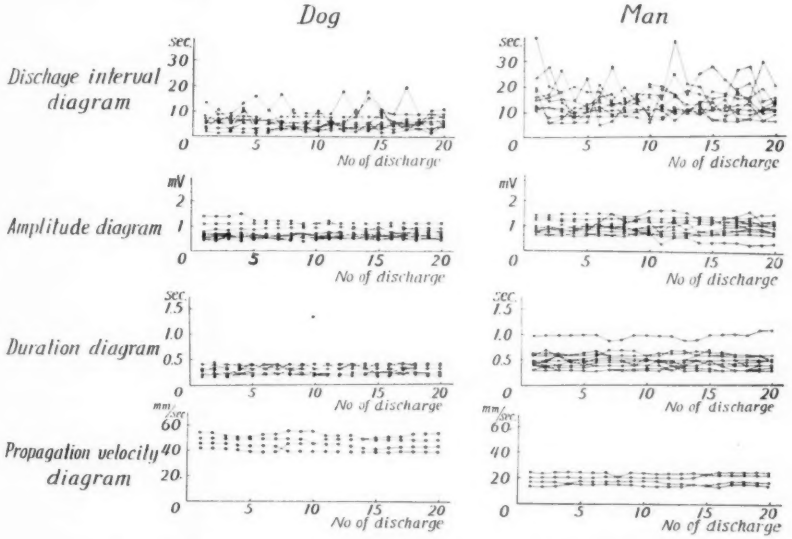

Fig. 7. Each curves in diagrams show progress of individual subject in dogs and men.

The duration of a discharge cycle changes in compliance with the change in wave patterns. In general, the duration ranged between 0.2 and 0.4 sec. in dog and between 0.3 and 1.1 sec. in man. The values were found usually smaller in the dogs than in the human subjects. As to the duration little temporary variations occurred over a very long period of recording (Fig. 7).

Measurements of propagation velocity. With bipolar leading we can

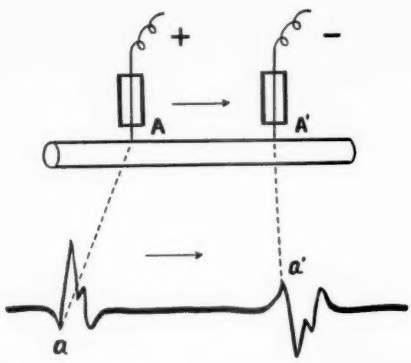

Fig. 8. Measurement of propagation velocity. V is propagation velocity (mm/sec). AA', distance between electrodes (bipolar) (mm). *a*, preceding positive deflection at A. *a'*, preceding positive deflection at A'. S, displacement speed of the recording paper in mm/sec.

measure the velocity of propagation. In Fig. 7, the proximallead is denoted by A, and the distal by A'. Denote the preceding positive deflection at A with *a* and that at A' with *a'*, then the propagation velocity V is given by the following formula:

$$V=\frac{AA'\times S}{aa'}$$

Where AA', *aa'* and S represent the distance between A and A' on the ureter, the distance from *a* to *a'* on the record, and the distance travelled by the recording paper per second respectively.

The measured values of propagation velocity were 38–56 mm./sec. in the dogs and 14–26 mm/sec, in the human subjects, the former showing a perceptibly higher velocity, but the change with time during any test of the same ureter, if any, was only very small (Fig. 7).

EUGs simultaneously recorded from ureters on both sides. With 10 dogs and 5 human patients as subjects, we made bipolar simultaneous recording of action potentials from both ureters of the same individuals and obtained such records as shown in Fig. 9. The wave forms of both potentials were similar in some cases but quite dissimilar in the others. The amplitude, the duration and the propagation velocity were always different in the right and left ureters, the intervals of discharges were also dissimilar, and we could not establish any antagonism between their potentials either.

Summary and Discussion

The authors recorded spontaneous action potentials of the ureters

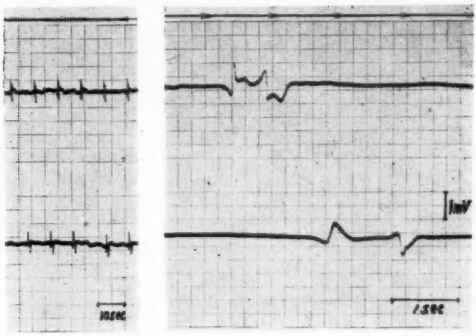

Fig. 9. EUGs simultaneously recorded from ureters on both sides of the same individual. Above, distance between electrodes is 25 mm. Below, distance between electrodes is 37 mm.

of dogs and human subjects and distinguished 3 different types of wave patterns. Our type I was found to coincide with that recorded by Butcher[11] from human subjects, by Sleator[10] from dogs and by Niu[7] from rats and cats, and our type II with that recorded by Orbeli and Brücke[1] and Aiba[3] from dogs, by Yano[5] from pigs, by Niu[7] from rats and by Obama[8] and Kudo[9] from cats. The slow curve recorded by Corey[12] from human ureters was of complicated rorm, but devoid of spiky elements. This is probably because it represents a mass response recorded with a large ring electrode affixed to a catheter, and it was another characteristic of this record that the phase reversal as found in usual records was not clear in his records obtained with bipolar leading. The wave forms of the continuous uretral discharges obtained by Baker[13] with a subcutaneous injection needle (No. 28) are different from ours in that they were triphasic, but showed considerable mutual dissimilarity.

As has been mentioned above, we succeeded in obtaining potentials of wholly identical wave form over a period as long as 3 hours, and this must be evidence that potentials provide a serviceable means in studying the function of the ureter.

Butcher has found uretral discharges coming forth in patterns of equal intervals or of groups, but we could also detect some cases of irregular occurrences.

In our present study, we obtained the measured values of discharge intervals of 1–20 sec. in dogs and of 5–40 sec. in men, of amplitudes of 0.5–1.5 mV in dogs and 0.3–1.7 mV in men, of duration of 0.2–0.4 sec. in dogs and 0.3–1.1 sec. in men and of propagation velocity, of 38–56 mm/sec in dogs and 14–26 mm/sec in men, values approximately in agreement with the values obtained by Butcher[11] with human, by Sleator[10] with canine

and by Niu[6)7)] with ratty and feline ureters.

Conclusion

The authors recorded the spontaneous action potentials of the ureters exposed under laparotomy of 12 human subjects with intestinal diseases but diagnosed as healthy in their urinary tract and of 42 normal adult dogs over a long period of 1–3 hours, to examine the properties of such potentials and arrived at the following conclusions.

1. The wave patterns were similar in dog and in man. The typical action potential consisted of four deflections, namely a preceding positive deflection, a major negative deflection accompanied by a minor one and a positive after-potential. This type was called Type I. The wave pattern lacking the minor negative deflection was designated Type II, the pattern apparently consisting of a main negative deflection alone was named Type III. All these potentials seemed to arise in the normal state of the ureter.

2. Discharge of potentials occurred either at regular and constant intervals, or in groups, or quite irregularly.

3. The intervals of discharge ranged between 1 and 20 sec. in dog and between 5 and 40 sec. in man, their amplitude between 0.5 and 1.5 mV in dog and between 0.3–1.7 mV in man, their duration between 0.2 and 0.4 sec. in dog and 0.3 and 1.1 sec. in man and the propagation velocity between 38–56 mm/sec in dog and 14–26 mm/sec in man. Both in man and dog, the discharge intervals changed only slightly with time, but almost no temporary change could be seen with other values such as maplitude, duration, etc. of such a change.

4. Simultaneous recording of the action potentials of ureters of both sides revealed no close correlation between the EUGs of both sides.

References

1) Orbeli & Brücke, Pflügers Arch., 1910, **133**, 341.
2) Hasama, *ibid.*, 1933, **232**, 236.
3) Aiba, Nisshin Igaku (Jap.), 1934, **23**, 965.
4) Bozler, Amer. J. Physiol., 1942, **136**, 553.
5) Yano, Tohoku J. Exp. Med., 1941, **41**, 13.
6) Niu, Nippon Seirigaku Zassi (Jap.), 1950, **12**, 377.
7) Niu, *ibid.*, 1951, **13**, 6.
8) Obama, Denki Seirigaku Kenkyu (Jap.), 1955, **7**, 59.
9) Kudo, *ibid.*, 1955, **8**, 67.
10) Sleator & Butcher, J. Physiol., 1955, **180**, 261.
11) Butcher & Sleator, J. Urol., 1954, **73**, 970.
12) Corey, Fite & Samuel, *ibid.*, 1956, **75**, 244.
13) Baker & Huffer, *ibid.*, 1953, **70**, 874.

The Tohoku Journal of Experimental Medicine, Vol. 69, Nos. 2-3, 1959

Toxic Substances in Cancer

VI. Effect on the Movement of Paramecium of the Extract of Gastric Carcinoma Tissue (2nd Report)

By

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(Received for publication, December 18, 1958)

In the first paper[1] it has been reported that the aqueous extract of gastric carcinoma tissue inhibits the movement of *Paramecium*, and that a basic substance of low molecular weight is responsible for this effect.

The present communication presents the results obtained from investigations on the extracts of the gastric carcinoma tissue with some organic solvents.

Experimental and Comments

Method

The strain of *Paramecium* used and the method for immobilization test were the same as described in the previous report[1].

In each material the immobilization titer, the highest possible dilution to immobilize *Paramecium* within 30 minutes, was measured.

Materials and Results

The test materials were prepared from the tissue of gastric carcinoma which was obtained by operation in our clinic. From the unaffected portion of the mucous membrane of the stomach with carcinoma (hereafter to be referred to as " non-carcinomatous portion " of the diseased stomach) and from the unaffected mucous membrane of the stomach with peptic ulcer (hereafter " normal gastric mucosa "), control materials were also prepared for comparison.

I. Extraction with alcohol

1) *Extraction.* About 10 g. of the tissue were minced, masticated in a mortar with a small amount of sea sand, put in 100 cc. of alcohol and left to stand for a week or more at room temperature. The extract thus obtained was separated by centrifugation, condensed under reduced pressure with nitrogen flow and dried to a brownish oily substance. Yields per 10 g. of the tissue were about 800 mg. in the case of gastric carcinoma

tissues, 650 mg. from non-carcinomatous portions and 700 mg. from normal gastric mucosae.

Results. See Table I.

TABLE I

Distribution of the Immobilization Titers of the Alcohol Extracts

Titer \ Materials	Diseased stomach		Normal gastric mucosa
	Carc. portion	Non-carc. portion	
800	2		
400	6	2	6
200	2	5	4
100		3	
Average titer	440	210	320

2) *Fractionation with acetone.* Five hundred mg. of the alcohol extract were rinsed with 10 cc. portions of acetone five times, 50 cc. in total, at room temperature. The remainder was dried (acetone-insoluble fraction, 380 mg. on an average). The rinsings were combined and dried after being condensed under reduced pressure (acetone-soluble fraction, average yield 70 mg.).

Results. As shown in Table II, immobilization titers were much higher in acetone-soluble fractions than in acetone-insoluble ones, both in cancer and in control cases.

TABLE II

Distribution of the Immobilization Titers of the Acetone-soluble and -insoluble Fractions

Titer \ Materials	Acetone-soluble			Acetone-insoluble		
	Diseased stomach		Normal gastric mucosa	Diseased stomach		Normal gastric mucosa
	Carc. portion	Non-carc. portion		Carc. portion	Non-carc. portion	
1600	3					
800	5	1	2			
400	2	5	6			
200		4	2	2		
100				2	2	4
0*				6	8	6
Average titer	960	360	440	60	20	40

* O means that the immobilizing activity is not perceptible even at 1 :100 dilution.

3) *Test with the acetone-insoluble fractions.* Acetone-insoluble fractions of the same tissue group were united and fractionated further by means of ether or chloroform, but no substance of the high titer was obtained.

II. Extraction with acetone

1) *Extraction.* Acetone extract was obtained from each tissue in a manner quite similar to the case of alcohol extraction. Mean yields from 10 g. of the tissue were 140 mg. in the case of carcinoma tissues, 100 mg. from non-carcinomatous portions and 100 mg. from normal gastric mucosae.

Results. See Table III. The differences between carcinoma materials and controls in the *Paramecium*-immobilizing activity were obvious.

TABLE III

Distribution of the Immobilization Titers of the Acetone Extracts

Titer \ Materials	Diseased stomach		Normal gastric mucosa
	Carc. portion	Non-carc. portion	
1600	4		
800	4		
400	1	1	3
200	1	2	3
100		4	1
0		3	3
Average titer	1020	120	190

2) *Systematic fractionation.* Acetone extracts combined in each tissue group were subjected to systematic fractionation for fat and fatty acids.[2] Thus, free fatty acids in the acetone extract were separated from neutral fats after neutralization, converted to lead salts and fractionated into two fractions: solid acid and liquid acid.

Results. As shown in Fig. 1 immobilization titers of the liquid acids were higher than those of other fractions. Of all the fractions, liquid acid of the gastric carcinoma tissue showed the most intense activity, its titer being 3200.

3) *Further fractionation of liquid acid of the gastric carcinoma tissue.* Two hundred and seventy mg. of the liquid acid fraction prepared from the tissue of gastric carcinoma were dissolved in 20 cc. of absolute alcohol. To the solution was added an equal volume of 0.05 per cent aqueous solution of lithium hydroxide and the mixture was cooled with ice. Crystalline precipitate here appeared was separated by filtration, purified by several successive recrystallizations and extracted with ether after being decomposed by hydrochloric acid (liquid acid I, yield 80 mg.). The filtrate and

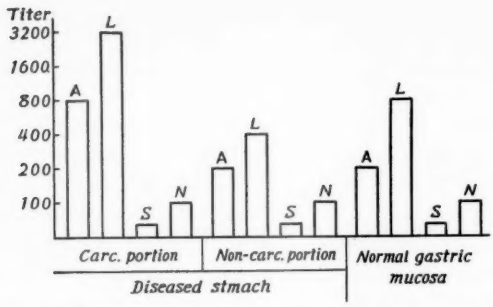

Fig. 1. Immobilization tests with the fractions : liquid acid (L), solid acid (S), and neutral fat (N). A, mother acetone extract.

the supernatants at recrystallizations, on the other hand, were united, the solvent was evaporated off and the residue was extracted with ether after acid decomposition (liquid acid II, yield 40 mg.).

Results. Immobilization titer of liquid acid I was 6400, higher than that of the mother liquid acid, while the titer of liquid acid II was as low as 400.

Paper chromatogram of liquid acid I. As shown in Fig. 2, liquid acid I gave a single spot of Rf 0.83 on a chromatogram, which was yellow by the treatment with bromcresol green and turned brown after the treatment with iodine, indicating that the fraction consists of unsaturated fatty acid.

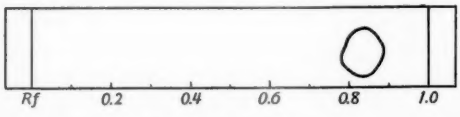

Fig. 2. Diagrammatic reproduction of the paper chromatogram of liquid acid I, obtained by one-dimensional descending irrigation with methanol-petroleum hydrocarbons (4:1). Paper, Toyo No. 2 filter paper, which was soaked in petroleum hydrocarbons (bp. 140–170°C). Temperature, 30°C. Duration, 6 hrs. Solvent front, 102 cm. Developer, alcoholic solution of bromcresol green and iodine-alcohol solution.

Summary

Alcohol extract, acetone extract and many of the various fractions of them prepared from the tissue of gastric carcinoma inhibited the movement of *Paramecium* more intensely than those from the control tissue.

Of all the fractions tested, liquid acid I which was fractionated from the acetone extract of the gastric carcinoma tissue was the most effective. The fraction consists of fatty acid, whose lithium salt is insoluble in 50 per cent alcohol.

References

1) Oh-Uti, Hasegawa, Yamaguchi & Abe, Tohoku J. Exper. Med., 1957, **67**, 87.
2) Oh-Uti, Abe & Yamaguchi, *ibid.*, the same volume, p. 91.

The Tohoku Journal of Experimental Medicine, Vol. 69, Nos. 2-3, 1959

Toxic Substances in Cancer

VII. Effect on the Excised Frog Heart

By

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(Received for publication, December 18, 1958)

Following Maki's investigation,[1] in which it was demonstrated that the aqueous extract of the tissue of gastric carcinoma contains a toxic substance inhibiting the excised frog heart, the authors studied the substances showing similar effect in the alcohol extract of the tissue, as well as in gastric juice of patients with gastric carcinoma.

Experimental with Comments

Method

The frog used was *Rana nigromaculata*, weighing 14 to 23 g. An excised frog heart was irrigated first with Ringer's solution for frogs, then with a test solution (Ringer's solution containing a test material at given concentration) and the changes in state of beats were recorded kymographically. Criterion for determining the effect of the material on the heart is shown in Fig. 1.

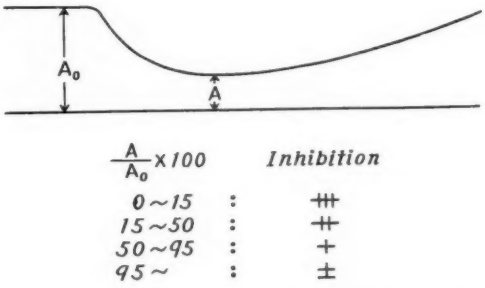


Fig. 1. Schema of a kymogram with the explanation of the signs showing the grade of inhibitory effect. A_0, amplitude before administration of the material; A, minimal amplitude after the administration.

Materials and Results

Fractions of the tissue of gastric carcinoma soluble in organic solvents

Portions of the materials, which the authors had prepared in the previous work[2] to test the effect on the movement of *Paramecium*, were used here.

The results are summarized in Table I. Of all the materials tested, liquid acid fraction of the gastric carcinoma tissue inhibited the heart most remarkably, showing marked differences in intensity from the corresponding fraction of the normal gastric mucosa (Fig. 2).

TABLE I

Inhibitory Effects of the Alcohol Extract and its Fractions of the Gastric Carcinoma Tissue on the Excised Frog Heart*

Material	Dilution (1:)							
	100	250	500	1000	2000	4000	8000	16000
Alcohol extract	### (###)	### (###)	+ (+)	+ (±)	± (±)	± (−)	− (−)	− (−)
Acetone soluble fraction	### (###)	### (###)	## (+)	+ (+)	+ (±)	± (−)	− (−)	− (−)
Acetone insoluble fraction	### (###)	+ (###)	± (+)	± (+)	− (−)	− (−)	− (−)	− (−)
Liquid acid	### (###)	### (###)	## (##)	+ (+)	+ (±)	± (−)	± (−)	− (−)
Solid acid	### (###)	### (###)	## (##)	+ (+)	+ (+)	+ (±)	− (−)	− (−)
Neutral fat	+ (###)	+ (##)	± (##)	± (+)	− (−)	− (−)	− (−)	− (−)

* Figures in parentheses show the results with the corresponding substances from the normal gastric mucosa.

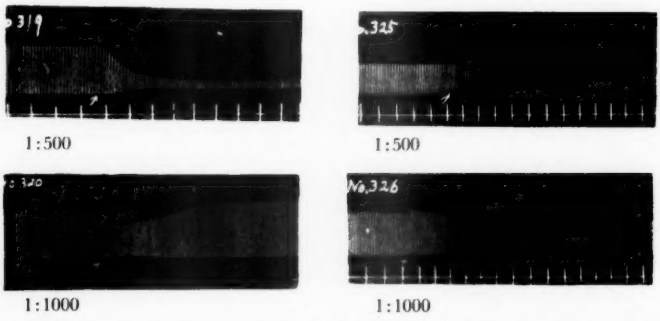


Fig. 2. Kymograms showing the effect of the liquid acid fraction obtained from the tissue of gastric carcinoma (left) or of normal gastric mucosa (right).

Gastric juice of patients with gastric carcinoma

1) Dried material. Gastric juices obtained from patients with gastric carcinoma were filtered, condensed under reduced pressure, dried to powder and combined together. The control material was prepared, in the same manner, from the gastric juices of patients with surgical diseases other than malignant neoplasms.

As shown in Table II, these dried materials inhibited the heart only at high concentrations.

Then, the dried materials were fractionated in a manner as shown in Table III and each fraction was tested for the effect on the heart. The results are summarized in Table II. No remarkably effective substance, as

TABLE II

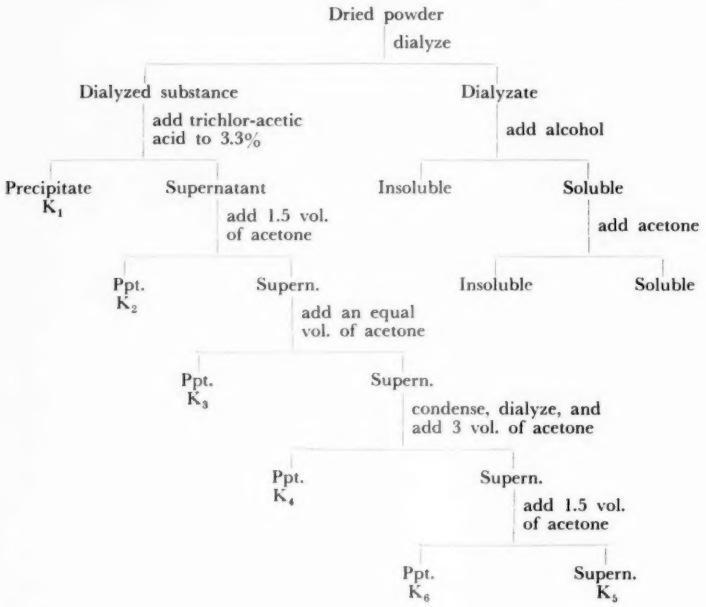
Inhibitory Effects of the Dried Powder and its Fractions of the Gastric Juice of Patients with Gastric Carcinoma on the Excised Frog Heart*

Material	Dilution (1:)				
	100	250	500	1000	2000
Dried powder	### (###)	### (###)	− (−)	− (−)	− (−)
Dialyzate	### (###)	+ (++)	+ (+)	− (+)	− (+)
Alcohol insoluble fraction	### (###)	+ (+)	+ (+)	− (+)	− (−)
Alcohol soluble fraction	### (###)	+ (###)	+ (+)	− (+)	− (−)
Acetone insoluble fraction	+ (###)	± (+)	− (−)	− (−)	− (−)
Acetone soluble fraction	### (###)	+ (++)	− (+)	− (±)	− (−)
Dialyzed substance	± (−)	− (−)	− (−)	− (−)	− (−)
K_1	++ (−)	+ (−)	− (−)	− (−)	− (−)
K_2	+ (+)	± (−)	− (−)	− (−)	− (−)
K_3	+ (−)	± (−)	− (−)	− (−)	− (−)
K_4	+ (−)	± (−)	− (−)	− (−)	− (−)
K_5	− (−)	− (−)	− (−)	− (−)	− (−)
K_6	+ (−)	+ (−)	− (−)	− (−)	− (−)

* Figures in parentheses show the results with the corresponding substances obtained from the gastric juice of patients with surgical diseases other than malignant neoplasms.

TABLE III

Scheme for Fractionating the Dried Powder of the Gastric Juice



much effective as the ether extract of fresh gastric juice which will be described below, was obtained by this method, though it was found that the fractions prepared from the dialyzate of the dried material were generally more intense in inhibitory effect than those derived from the dialyzed substance.

2) Ether extract. Gastric juices were fractionated systematically in the manner in which the authors had previously followed after the *Paramecium*-immobilizing substance in the gastric juice of patients with gastric carcinoma.[3)]

An outline of the method is as follows: Fresh gastric juice was agitated in a fractionation funnel with an equal volume of ether and the ether extract was obtained by drying the upper ethereal layer by the aid of a fan. Pooled ether extract was then treated with acetone to be fractionated into soluble and insoluble fractions. A portion of the acetone-soluble fraction was neutralized with sodium methoxide and separated from neutral fat. Fatty acids thus isolated were then converted to lead salts and divided into two fractions: liquid acid, whose Pb salt is insoluble in alcohol, and solid

acid, whose salt is soluble in this solvent.

In Table IV are shown the effects of these fractions on the excised frog heart. Ether extract of the gastric juice of patients with gastric carcinoma was much more effective than the control extract, the highest possible dilution of the former to inhibit the heart being eight times as much as that of the latter.

TABLE IV

Inhibitory Effects of the Ether Extract and its Fractions of the Gastric Juice of Patients with Gastric Carcinoma on the Excised Frog Heart*

Material	Dilution (1:)								
	100	250	500	1000	2000	4000	8000	16000	32000
Ether extract	### (###)	### (###)	### (##)	## (+)	+ (±)	± (−)	± (−)	± (−)	− (−)
Acetone soluble fraction	### (###)	### (##)	### (+)	+ (−)	+ (−)	± (−)	± (−)	− (−)	− (−)
Acetone insoluble fraction	+ (+)	+ (+)	± (−)	− (−)	− (−)	− (−)	− (−)	− (−)	− (−)
Liquid acid	### (###)	### (###)	## (+)	+ (+)	+ (+)	+ (±)	± (−)	± (−)	− (−)
Solid acid	### (###)	## (##)	+ (+)	+ (+)	± (+)	− (−)	− (−)	− (−)	− (−)
Neutral fat	### (###)	+ (+)	+ (+)	+ (+)	± (±)	− (−)	− (−)	− (−)	− (−)

* Figures in parentheses, see Table II.

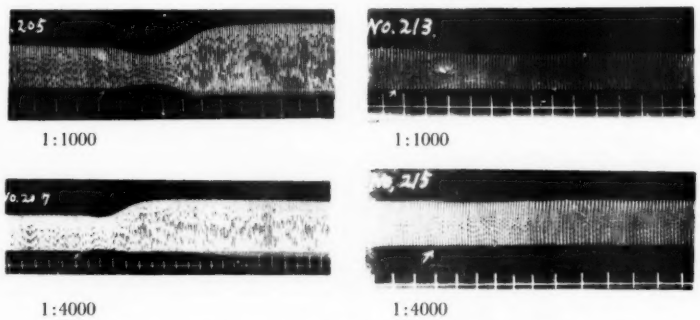


Fig. 3. Kymograms showing the effect of the liquid acid fraction separated from the gastric juice of patients with gastric carcinoma (left) or of patients with surgical diseases other than malignant neoplasms (right).

Effective substance in the ether extract of cancer material was soluble

in acetone. Of the three materials prepared from the acetone-soluble fraction of the extract, liquid acid fraction presented the most intense inhibition, more marked at various concentrations than that of the corresponding control fraction (Fig. 3).

SUMMARY

1. Liquid acid fraction isolated from the alcohol extract of the tissue of gastric carcinoma inhibited the excised frog heart more markedly than the corresponding fraction of the normal gastric mucosa.

2. Dried powder of gastric juice of patients with gastric carcinoma and many of its fractions showed only slight inhibition to the heart.

3. Ether extract of fresh gastric juice of patients with gastric carcinoma inhibited the heart intensely while the control extract was far less effective. Of all the fractions of the extract, liquid acid fraction was the most effective in inhibition.

References

1) Maki, Tohoku J. Exper. Med., 1956, **63**, 121.
2) Yamaguchi, Kamma & Oh-Uti, *ibid.*, this volume, p. 185.
3) Oh-Uti, Abe & Yamaguchi, *ibid.*, 1957, **67**, 91.

The Tohoku Journal of Experimental Medicine, Vol. 69, Nos. 2-3, 1959

Effects of Orally Administered "Tween 80" on the Mesenteric Lymph Nodes of Intact and Adrenalectomized Mice

By

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(Received for publication, December 29, 1958)

Since Flemming[1] has discussed on the functional significance of the secondary nodules in the lymph node in his comprehensive studies on the regeneration of tissue, very many works on the function and structure of the lymph node and lymphatic tissue have been published by a number of investigators. A considerable part of these works has been carried out by following up, histologically or cytologically, the structural changes produced by various kinds of stimuli. In this study, observations on the histological alterations in the mesenteric lymph nodes of mice caused by an oral administration of an emulsifying agent, polyoxyethylene sorbitan mono-oleate (Tween 80), are presented.

On the other hand, on the basis of recent endocrinological observations, it is well-known that stimulations of wide variety invariably produce an acute involution of lymphatic organs through enhanced pituitary-adrenocortical secretion. Agents causing such an adrenocortical response of lymphatic organs have been reviewed by Dougherty and White[2] and Selye[3]. As has been stated by Dougherty[4], therefore, results of numbers of previous experiments on the lymphatic tissue may be deemed to be influenced in part by the destructive effect of hypersecretion of adrenocortical hormones induced by the administration of exogenous stimuli. Thus, in this study, a group of animals used had been adrenalectomized prior to the experiment, in order to make the essential effects of Tween more evident, avoiding lymphocytolytic effect of adrenal corticoids.

Experimental

Materials and Methods

As materials, dd-strain female mice of 5–6 weeks old were used, which were divided into two groups of I and II. Each animal of the former group had been kept intact until 0.4 ml. of 20% aqueous solution of Tween 80 was in-

troduced into the stomach through a gastric tube, while animals of the latter group had been adrenalectomized one week in advance of the administration of the same dose of Tween. From the day following the adrenalectomy, daily dose of 25 μg./10 g. body weight cortisone acetate (Cortone "Merck") was subcutaneously injected to each animal of the second group till the second day before the administration of Tween, after which 12.5 μg./10 g. body weight/day of Cortone was given until the animal was sacrificed. These doses of cortisone were decided according to Santisteban and Dougherty[5] who estimated the daily dose of cortisone required to maintain the weight of the thymus of adrenalectomized CBA mouse at the level of intact mouse to amount 25–50 μg./18 g. body weight.

Two or three mice each of both the groups were sacrificed at 1, 3, 6, 12, 18, 24 hours and 2, 3, 4, 6 and 8 days after the administration of Tween, and the mesenteric lymph node of each animal was fixed with Zenker's fluid or Zenker-formol, imbedded in paraffin, and cut into 5–7 μ sections. They were then stained with hematoxylin and eosin, hematoxylin and Giemsa, May-Grünwald and Giemsa, and methyl green and pyronin.

Observations

1) Group I (intact-Tween administered animals). From 1 hour after the administration of Tween, marked decrease of lymphocytes, chiefly of small-sized ones, was noticed, and it was gradually intensified to reach a very high degree by lapse of time. Within 12 or 18 hours, the cortical lymphoid tissue came to contain mainly reticulum cells, lymphoblasts and immature lymphocytes of various sizes in residue, resulting in a very diffuse and loose appearance of the lymphoid tissue (Fig. 1). Besides large quantity of nuclear debris contained in the secondary nodules, many pycnotic nuclei and nuclear fragments were found scattered about in the whole area of the cortex, suggesting that a considerable part of the depletion of lymphocytes was due to their destruction.

Often small necrobiotic areas and diffuse infiltration of granulocytes were found in the cortical lymphoid tissue. The endothelial cells of the small vein in the cortex were sometimes highly hypertrophied into tall cubic form. Coinciding with the description of Ehrich[6], in the lumina of these post-capillary veins numerous small lymphocytes were frequently crowded, in striking contrast to the pre-capillary arterioles containing nearly nothing but erythrocytes.

As shown in Fig. 1, the depletion of lymphocytes took place also in the dense marginal zone of small lymphocytes, the so-called "corona", circumscribing the light center of the secondary nodule. So that, most of the secondary nodules came to be utterly lack of their lymphocyte walls leaving the light centers denuded. Such a light center consisted mainly of cord-like aggregation of large-sized immature lymphocytes with rather weakly

basophile cytoplasm, that is, lymphoblasts in the broad sense or the " meso-lymphocytes " of Maximow[7),8)]. But rather rarely any of these cells were found in mitotic division, and numerous macrophages ingesting large quantity of nuclear debris were found between the cords of these cells. This finding may clearly represent an accerelated destruction of lymphocytes accompanied with severe suppression of lymphocytopoiesis in these centers, suggesting that such a formation may be assumed as the " reaction center " of Hellman.[9)]

In the specimens sampled at 12–72 hours of experiment, these bare reaction centers tended to be found in the deeper part of the lymph node, i.e., nearer to the medulla. Some of these deep-situated centers had irregular circumferences, and judging from rather loose arrangement of the reticular fibers surrounding them, they are no longer exerting any strong expansile pressure on the surrounding cortical tissue. Besides, in the deep part of the cortex adjacent to the medulla of 72 hour-specimens, the cords of lymphoblasts of some of these light centers were found broken up into pieces, indicating a dissolving process of the centers. On the other hand, the secondary nodules ranged in the superficial zone of the lymph node were covered by more or less dense walls of small lymphocytes, especially in the peripheral side facing the marginal sinus. These nodules were often in the shape of a long ellipsoid, taking their longer axes perpendicularly to the surface of the lymph node. In the specimens of 48–72 hours, some of them became much elongated reaching the vicinity of the cortico-medullary junction, and showed the appearance as if the lumps of lymphoblasts were pushed out from the deeper pole of the nodules (Fig. 2). Such a finding is in good agreement with the destructive phase of secondary nodules described by Horii and Shimada[10)] and Shimada[11)]. In these light centers covered with lymphocyte walls too, the same cellular constitution, inhibited proliferation and accentuated phagocytosis, as found in the bare reaction centers was observed.

The decrease of mature lymphocytes was observed not only in the cortical region, but also in the medullary cords, where the density of cells was reduced and above all small lymphocytes were scanty, but large cells with evidently basophile cytoplasm remained apparently unaffected. These basophile cells were sometimes ranged on the surface of the medullary cords facing the sinuses.

Some of them exhibited largely expanded, intensely basophile cytoplasm surrounding light-staining vesicular nuclei in which one or two large angular nucleoli were visible. These cells may be looked upon as belonging to the type of very immature lymphoblasts or " lymphogonias " of Amano *et al.*[12)–14)], and were found sporadically not only in the medullary cords, but also in the whole area of the cortex, and in small numbers in

the light centers of the secondary nodules.

The remainder of the basophile cells in the medullary cords were mostly smaller in size, and the nuclei in them stood somewhat excentrically. Except in the cell-center region, the cytoplasm of these cells showed such an intense basophilia that they stained dark blue with May-Grünwald-Giemsa, while those of the lymphoblasts were colored sky-blue in the same sections. These cells may be decided to belong to the series of plasma cells, rather than to that of lymphocytes, and as representing the various stages of maturity of the former cells. Coinciding with the description of Amano *et al.*, their distribution was fairly limited to the medullary cords and the vicinity of the cortico-medullary junction, especially in the peripheral part of the parenchyma facing the sinuses. However, large, immature form of these plasma cells were not always easy to distinguish them from the lymphoblasts. Slight infiltration of granulocytes was also observed in the medullary cords of some specimens, especially in the early stage of the experiment.

Four days after the administration of Tween, mature lymphocytes were found considerably restored in the whole parenchyma including cortex and medulla. At this time, nuclear debris was almost disappeared from the light centers, most of which were surrounded by dense marginal walls of small lymphocytes, and numerous mitotic figures were found in the cortical lymphoid tissue as well as in the secondary nodules. But such an increase of the lymphocytes reached the maximum on the 4th day of the experiment, and on the 6th day, the lymphocytes again began to decrease. The secondary nodules came to be devoid of lymphocyte walls, and the light centers resumed the appearance of being reaction centers.

In addition to the histological changes in the parenchyma described above, the findings on the sinus must also be noted. The mesenteric lymph nodes are presumably under stimulation of the digestive tube in normal time too, and are often found containing a considerable amount of lymphocytes including immature ones of more than medium size in their intermediate and medullary sinuses. However, a prominent corwding of small lymphocytes in the sinus illustrated in Fig. 3, showing the medullary portion of a specimen sampled 1 hour after the treatment, is obviously exceeds normal range of cellular density in the sinus. In this figure, in addition, it should be noticed to the very even dissemination of lymphocytes throughout the parenchyma and sinus, which obscures the boundary between these two structures. This may be regarded as a sign of accerelated emigration of the small lymphocytes from the former into the latter. Most specimens later than 3 hours also showed slighter but perceptible crowding of lymphocytes mainly in the intermediate sinuses and partially in the medullary sinuses adjacent to the former. The parts of sinuses where no lymphocyte

was contained were sometimes found nearly filled up with hypertrophied reticulum cells. Such a figure may be decided as a sign of so-called sinus catarrh or sinus reticulosis. In the sinuses of some specimens, megakaryocytes were found. Though it was rare, a limited number of neutrophile and eosinopile myelocytes were also found.

2) *Group II* (*adrenalectomized-Tween administered animals*). The main findings here consisted in the frank depletion of the mature lymphocytes in the first 48 hours after the administration of Tween, and the recovery in density of the cells on the 4th day, and their redecrease on the 6th day, keeping a fairly parallel fluctuation with similar findings in Group I. However, evident differences were found between the findings on the mesenteric lymph nodes of the animals in Group I and those in Group II, and on such points of dissimilarity, we will mainly describe in the following.

In many cases of this group too, the marginal walls of the secondary nodules were found thinned away and nearly gone out altogether. But our attention was attracted to the fact that the destruction of lymphocytes in the light centers was seen milder in the major cases of this group than in the first group, and especially few macrophages that ingesting nuclear debris were found in our specimens sampled within the first three hours following the treatment. After the 6th hour, the destruction of lymphocytes in the light centers became fairly perceptible, but seemed yet far milder than in Group I (Figs. 4 and 5).

On the other hand, the proliferation of the lymphocytes was apparently not so much inhibited as in Group I. The lymphocyte walls of the secondary nodules were found in reappearance sometimes in the 18 hour-specimens and generally in the 24 hour-specimens. Here, quite as in Group I, the marginal walls were frequently open towards the deeper, i.e., medullary side, taking a cap-like form covering the superficial, i.e., marginal side of the light centers. In some cases, the appearance was shown as if the lumps of immature lymphocytes in the light centers were pushed out through the open side of the wall towards the medulla.

The karyokinetic figures of lymphocytes increased in 18 hours or later, and in some specimens at 24–48 hours, small bare light centers began to appear at the cortico-medullary junction or in the medullary cords. These centers were composed of lumps of immature large lymphocytes, some of them were in mitosis, and scarcely contained any macrophages (Fig. 6). Therefore, these are not denuded reaction centers described above, but are conceivably just the formations called by Conway[15] as " bare germinal centers." No evidence has been proved, however, that these small germinal centers grow up to large secondary nodules in the later stage of the experiment.

The medullary cords were often highly swollen and sometimes charged

with strongly basophile cells, but among these cells, those apparently belonging in the plasma cell-series seemed more scarce than in Group I.

The finding further to be noted in this group was the large accumulation of lymphocytes in the intermediate and medullary sinuses. This accumulation was more intense than in Group I, and was found throughout whole time of observation, accompanying some rises and falls in its intensity. In particular, upon examination under low magnification of the early stage specimens, we could see some sinuses filled up with crowds of lymphocytes were standing out by their darkness against rather pale parenchyma (Figs. 5 and 7).

In these adrenalectomized animals too, megakaryocytes were often found in the sinuses, but retention of myelocytes was observed only scarcely. While in these young adult mice, myeloid tissue still remains in the red pulp of the spleen, some animals have been also splenectomized at the time of adrenalectomy, in order to determine the origin of these myeloid elements found in the lymphatic sinuses. In the mesenteric lymph nodes of these adrenalectomized-spelnectomized mice, megakaryocytes were also found, and a few specimens, moreover, showed remarkable aggregation of myelocytes in some medullary cords. This finding suggests not only that some of these myelogenous cells originate from the bone marrow, but also that these cells emigrated out from the bone marrow have probably been filtered out from the blood stream by the spleen in not splenectomized mice.

Discussion

Thanks mainly to the works of Dougherty and White[2),16)], as stated in the introduction, it is well-known that hypersecretion of adrenal cortical hormones results in an acute dissolution of small lymphocytes and subsequent general involution of lymphatic tissue. In particular, on this subject, comprehensive reviews have already been made by White[17)] and Dougherty[4)], so that it would be needless for us to resume a general discussion about it.

On the other hand, numerous experimental studies have been carried out under such strong stimulating as injection of vaccines or toxic substances, and ionizing irradiation, all of which must be acknowledged as capable of acting as " stressor " on the living organism. As was stated by Dougherty[4)], in such experiments used those noxicious stimuli, it cannot exclude destructive effect of stress on the lymphatic organs mediated by adrenal corticoids. In this connection, we have only to notice a report of Dougherty and White[18)], who demonstrated that rather small amount of stimulus (X-ray irradiation amounts 10 r.) is sufficient to induce hypersecretion of adrenocortical hormones.

In our study too, the mesenteric lymph nodes in the adrenalectomized mice survived by administration of cortisone were found not only hypertrophied in general, but even after Tween administration, showed milder lymphocytolysis and slighter inhibition of lymphocytopoiesis. This finding is in good agreement with Dougherty and White[16], and also with what has been reported on adrenalectomized animals. Therefore, the drastic degeneration of lymphocytes observed in intact-Tween administered mice seems to suggests an augmentation of adrenal cortical secretion in them. This assumption is supported by Kato's observation[19] on the inguinal and axial lymph nodes and the white pulp of the spleen in the same experiment. He found vigorous cell-destruction in all of these lymphatic organs of intact-Tween administered mice within 1 hour after the treatment, while those of adrenalectomized mice remained apparently normal until 3 hours after the similar administration of Tween. In the mesenteric lymph nodes of the adrenalectomized mice too, degeneration of lymphocytes was observed in a somewhat later stage in a lower extent. In this case, it may be infered that Tween itself acts harmfully on lymphocytes, especially on small mature ones. The mechanism of such a lymphocytolytic effect of Tween remains obscure.

At any rate, one of the principal changes took place in the lymph nodes in animals of both the groups was the destruction and subsequent regeneration of lymphocytes, that is a finding being not essentially different from the results obtained in many experiments reported in the past.

Many years ago, Flemming[1] gave the functional appellation of " germinal centers " to the secondary nodules assuming this structure as a center of lymphocytopoiesis. But later on, this germinal center theory had been challenged by Hellman[9] who advocated rather to call them " reaction centers ". Since then, the secondary nodules have been most extensively studied among all the structures in the lymphatic tissue. The numerous past reports, moreover, brought it evident that secondary nodule is not a permanent structure, but is inconstant formation undergoing a cycle of various morphological and functional phases. For example, the description of Maximow[7,8] is a thorough account of the morphological variations of the secondary nodules based on the germinal center theory. Such cyclic changes were studied by Ehrich[6] and Conway[15] too, and Ehrich's classification has been recently supplemented by Shimada[11] in further details.

Horii and his co-workers[10,11,20,21] have also made detailed studies on the functional structure of the lymph node. Taking the difference between the number and maturity of lymphocytes appearing in the afferent lymphatics on the one hand, and those in the efferent lymphatics on the other as the index of the function (production and release) of each lymph

node, they tried to study it in comparison with the histological findings. Their results showed that the Flemming's secondary nodules did not directly participate in the production of lymphocytes, but upon their collapse involving dissemination of lymphoblasts packed in the light centers into the cortical lymphatic tissue, the production of lymphocytes was rapidly enhanced in the cortex. Their opinion well agrees with that of Ehrich, who looked upon the Flemming's secondary nodules as "storing places or reserve depots of so-called lymphoblasts."

In this study too, in concurrence with Horii *et al.*'s findings, the specimen sampled on the 2nd or 3rd day of the experiment from both the groups of intact and adrenalectomized mice, the lumps of lymphoblasts in the light centers were found pushed out towards the medulla blurring their boundaries. On the 4th day following such changes, the density of small lymphocytes began to rise in the lymphatic tissue. But it was noticed in this study, that not every light center was found to be dissociated, but considerable number of them were seemed to remain as the bare light centers in the deep area of the node, and regain their dense marginal walls of small lymphocytes.

The most important ground on which the germinal center theory stands is naturally the abundance of karyokinetic figures in the secondary nodules. Thus, it is inevitable that this theory faces a serious criticism when secondary nodules are found to have no lymphocytes in mitosis in them under some conditions. But in such conditions as the lymphocytopoiesis is entirely suppressed in the secondary nodules shown in many previous publications and in our intact-Tween animals, it cannot be denied the increased secretion of adrenal corticoids which react inhibitory on the proliferation of lymphocytes. Another support for this assumption may be given by constant existence of karyokinetic figures in the secondary nodules of adrenalectomized animals as is observed in this study. Then, we rather consider the secondary nodules as a site of active lymphocytopoiesis, though they are by no means the largest center of the proliferation.

Another finding in our experiment is the enhanced release of the lymphocytes into the sinus, which was more frankly observed in Group II. Horii says that there are two modes of lymphocytosis in the efferent lymphatics, the one due to expedited production of lymphocytes in the lymph nodes (his so-called production lymphocytosis) and the other due to enhanced release of lymphocytes without adequate production (mobilization or emigration lymphocytosis). The release of lymphocytes in the early stage of our experiment in Group I seems to be something approximating the second state above. The finding in Group II was not merely of that mobilization, but here mobilization and proliferation were both in action.

The release of lymphocytes observed in both the groups of mice may be deemed to represent one of the effects of Tween. Whether the specific surface phenomenon of Tween solution as an emulsifying agent is responsible for the release of cells awaits further study. But we must call our attention to the fact that Tween has been administered *per os* in this experiment, so that some parts of Tween should be hydrolyzed by intestinal and pancreatic esterases before it was absorbed through intestinal wall. Nevertheless, it is of interest that Osogoe and Ōmura[22)-24)] have found conspicuous release of immature blood corpuscles from the bone marrow of rabbit which received intravenous injection of lethal dose of saponin, a substance equally exhibiting a character as a surface active agent. As for the mechanism of such a discharge of bone marrow cells into blood stream, Ōmura and Osogoe said that an injury to the endothelial walls of the capillary sinusoids resulting in extensive marrow hemorrhage must first be considered, and the depletion of the marrow elements proceeded parallel to the marrow hemorrhage. They found in their experiment a similar injury to the endothelial walls of the venous sinuses in the spleen and those of the medullary sinuses of the lymph nodes, too. Some of our specimens were silver-impregnated according to the method of Foot-Foot, but no structural alterations in fibrous reticulum of walls of sinuses revisable for clarifying the mechanism of release could be obtained. In our specimens too, some myeloid elements were not rarely found out. Our observation on adrenalectomized and splenectomized mice proved that administration of Tween accerelate the emigration of the myeloid cells from the bone marrow. But we cannot assume the bone marrow alone to constitute the origin of the myelogenous cells, because in such young adult mice as used in this study, myelogenic hemopoiesis is still in progress in the red pulp of the spleen.

Summary

In this study, histological observations on the structural alterations caused in the mesenteric lymph nodes of young adult female mice after an oral administration of Tween 80 were presented. Some of the animals were preliminarily adrenalectomized, in order to prevent destructive influence of adrenal corticoids on the lymphatic tissue. A comparison between the changes observed in the mesenteric lymph nodes in both the groups of intact and adrenalectomized animals has led to the conclusion that Tween accerelates the release of lymphocytes from the parenchyma of the lymph nodes into the sinus, and besides, that Tween may possibly act destructively on the mature lymphocytes.

References

1) Flemming, Arch. mikr. Anat., 1885, **24**, 50, 72, 355.

2) Dougherty & White, J. Lab. Clin. Med., 1947, **32**, 584.
3) Selye, Stress, The physiology and pathology of exposure to stress, Acta Inc. Med. Publ., Montreal, 1950, 452.
4) Dougherty, Physiol. Rev., 1952, **32**, 379.
5) Santisteban & Dougherty, Endocrinol., 1954, **54**, 130.
6) Ehrich, Am. J. Anat., 1929, **43**, 347.
7) Maximow, Möllendorffs Hdb. mikr. Anat., Springer, Berlin, 1927, II/I, 335.
8) Maximow, Special Cytology (Ed. by Cowdry), Hoeber, New York, 1932, **2**, 603.
9) Hellman, Möllendorffs Hdb. mikr. Anat., Springer, Berlin, 1930, VI/I, 232.
10) Horii & Shimada, Sogo Rinsho (Jap.), 1956, **5**, 1261.
11) Shimada, Rep. 2nd Div. Anat. Dept. Kyoto Univ. (Jap.), 1956, **4**, 183.
12) Amano, Unno, Hanaoka & Tamaki, Acta Path. Jap., 1951, **1**, 117.
13) Unno & Hanaoka, Symp. on Hematol. (Jap.), 1954, **6**, 62.
14) Unno, Hanaoka, Iwai, Hashimoto & Morita, Acta Path. Jap., 1954, **4**, 75.
15) Conway, Anat. Rec., 1937, **69**, 487.
16) Dougherty & White, Am. J. Anat., 1945, **77**, 81.
17) White, The Harvey Lect., 1947–48, **43**, 43.
18) Dougherty & White, Endorcinol., 1946, **39**, 370.
19) Kato, Teishin Igaku (Jap.), 1959, **11**, (in press).
20) Horii, Arb. 3ten Abt. Anat. Inst. Univ. Kyoto, Ser. D, 1938, **7**, 121.
21) Horii & Tamaki, Studies on the Lymphocytes (Jap.), Nippon Isho Shuppan, Tokyo, 1951.
22) Osogoe, Symp. on Hematol. (Jap.), 1949, **2**, 189.
23) Ōmura, Acta Haematol. Jap. (Jap.), 1950, **13**, 284.
24) Ōmura & Osogoe, Anat. Rec., 1951, **110**, 289.

Plate I

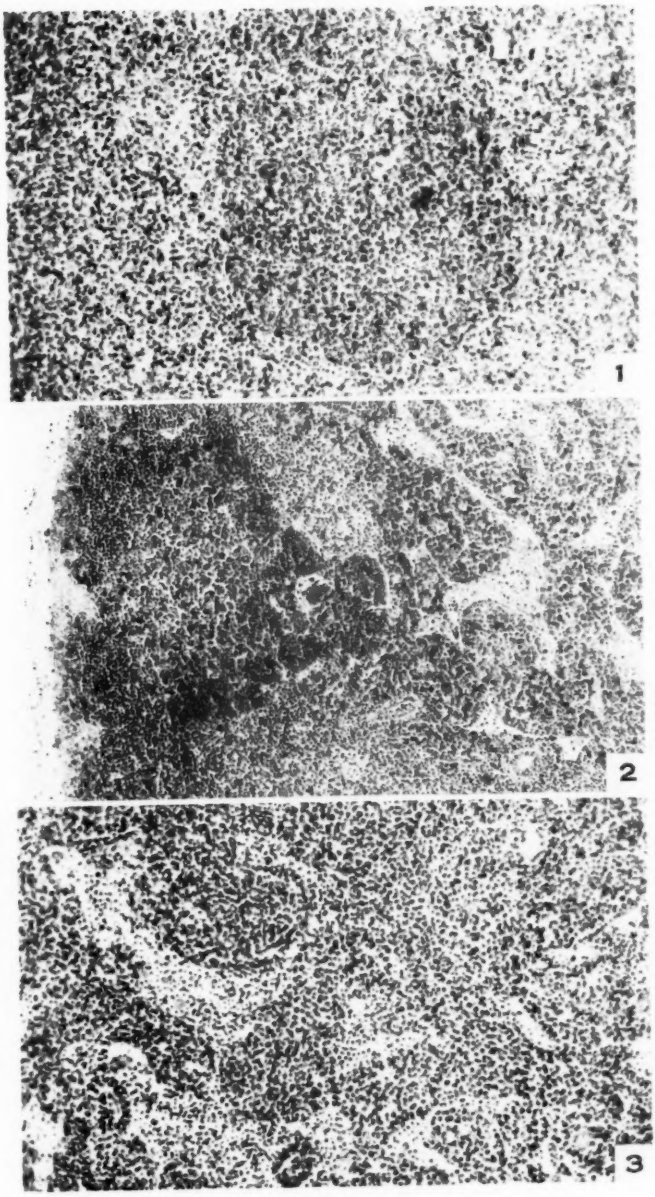

Plate II

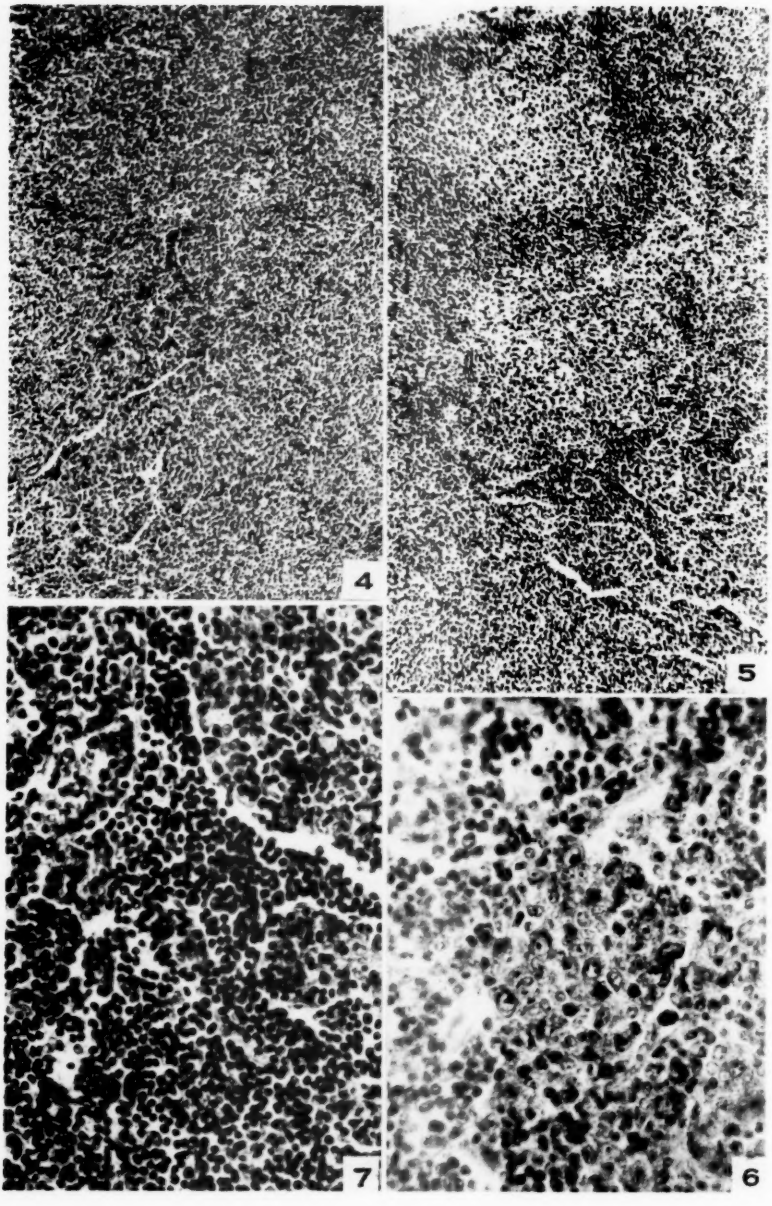

Plate I. Figures in this plate show sections of the mesenteric lymph nodes of intact-Tween administered mice.

Fig. 1. A reaction center devoid of marginal zone in the cortex of the lymph node sampled 18 hours after the administration of Tween. Numerous nuclear fragments are visible in the center. Cortical lymphoid tissue looks very loose. Stained with May-Grünwald-Giemsa. ×140.

Fig. 2. Topographical view of the lymph node sampled 72 hours after the treatment with Tween. A lump of the lymphoblasts is pushed out from the deep pole of the secondary nodule. The medullary sinus is partially filled up with hypertrophied reticulum cells. Stained with May-Grünwald-Giemsa. ×100.

Fig. 3. Medullary portion of the lymph node sampled 1 hour after the treatment with Tween. Even dissemination of small lymphocytes throughout medullary cords and sinus is shown. ×140.

Plate II. Figures in this plate show the mesenteric lymph nodes of adrenalectomized-Tween administered mice.

Fig. 4. Topographical view of the lymph node sampled 1 hour after the treatment with Tween. Decrease of small lymphocytes from the marginal walls of secondary nodules and from the cortical parenchyma, and their accumulation in intermediate sinuses are shown. Stained with hematoxylin and eosin. ×100.

Fig. 5. Topographical view of the specimen sampled 12 hours after the treatment. Intermediate sinuses are extensively dilated and packed with small lymphocytes. Stained with hematoxylin and eosin. ×100.

Fig. 6. A small bare germinal center in the medullary cord of a specimen, 48 hours after the treatment. Medullary sinuses are also filled up with many lymphocytes of various sizes. Stained with May-Grünwald-Giemsa. ×400.

Fig. 7. Cortical region of the same specimen as Fig. 6, showing prominent accumulation of small lymphocytes in the intermediate sinuses. Many mitotic figures are visible in bare light centers (upper right and left). ×250.

The Tohoku Journal of Experimental Medicine, Vol. 69, Nos. 2-3, 1959

Beiträge zur Histologie der Nebennierenrinde und der akzessorischen Nebenniere mit besonderer Rücksicht auf das sog. „androgene Gewebe“*

Von

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(Received for publication, January 5, 1959)

Wie bekannt ist bei menschlichen Feten der innere Teil der Nebennierenrinde schon in der 8. Fetalwoche als sog., „Fetalzone“ morphologisch vom übrigen Rindengewebe abgsondert, die während und nach der Geburt wenigstens zu einem grossen Teil der Involution anheimfällt.

Howard-Miller[1] wies auf das Bestehen gewisser Ähnlichkeiten in Bezug auf die Degenerationsprozesse hin, die in der Fetalzone des Menschen einerseits und in der X-Zone der Mäusenebenniere andererseits vor sich gehen. Waring[2] betrachtet auch beide Gewebe als wahrscheinlich homolog.

Über dies Problem machte Grollman eingehend Mitteilung in seiner Monographie (1936)[3]. Nach ihm besteht die NNR des menschlichen Fetus aus zwei funktionell voneinander abtrennbaren Teilen. Der eine, welcher mit dem Mark in direkter Berührung steht, soll dasjenige Inkret produzieren, dem die vermännlichende Wirkung der NNR zuzuschreiben sei, und wird von ihm als androgene Zone bzw. Gewebe bezeichnet. Dies Gewebe soll nicht nur funktionell sondern auch morphologisch und histochemisch vom übrigen Rindenabschnitte—dem für Lebenserhaltung not-

* Die vorliegende Arbeit, die durch Grollman'sche Veröffentlichung[3] über das Wesen und die Bedeutung der X-Zone der Maus und der Fetalzone des Menschen angeregt aufgenommen wurde, war schon in Jahre 1943 fertig gemacht worden, hatte aber aus äusseren Gründen bis jetzt keine Gelegenheit zum Abdruck gebracht zu werden. Wie bekannt, haben die Biologie und Morphologie der Nebenniere in neuerer Zeit merkwürdige Fortschritte gemacht. Auch Grollman hat gegenüber seinen früheren Angaben gewisse Änderung vorgenommen (Gersh u. Grollman, Anat. Rec., 1939, **75**, 131). Da aber das Problem heute noch keineswegs zum Schluss gekommen ist und die Ergebnisse dieser Arbeit, die natürlich in einem Teil ihre Bedeutung eingebüsst haben, auch heute in mancher Hinsicht immer noch neue Kenntnisse darbieten, so dürfen sie hier in kurzen Zügen mitgeteilt werden. Die angeführten Literaturen sind nur auf die damals zitierten beschränkt.

wendigen Teil, d.h. „cortical tissue proper" nach diesem Autor—sondergestellt werden. Ferner erwähnt er, dass die X-Z der Maus mit der Fetalzone des Menschen homolog sei. Das wesentlichste Charakteristikum beider Gewebe steht darin, dass sie nur in einer kürzeren Zeitperiode in Erscheinung treten. Vines[4] erörtert die Beziehung zwischen der von ihm gefundenen fuchsinophilen Substanz und der androgenen Wirkung des Rindengewebes.

Nach Aichel[5] lassen sich unter den akzessorischen Nebennieren diejenigen, die sich kaudal vom unteren Nierenpol besonders im Gebiete der inneren Genitalien vorfinden, genetisch davon unterscheiden, die in der Nähe des Hauptorgans vorhanden sind.

Unter Heranziehung dieser Schilderungen Aichel's, wogegen schon von Pick[6] und Poll[7] die kritische Stellung genommen worden ist, schreibt Grollman, dass die akzessorsichen Rindenknötchen abgesehen von denjenigen, die sich in der direkten Nähe des Hauptorgans befinden, grösstenteils nicht anders als das sog. androgene Gewebe sind, welches vom „cortical tissue proper" bzw. „true accessory cortical tissue" funktionell völlig sonderzustellen ist. Bei Kaninchen verleiht die akz. NN, ohne Unterschied ihres Sitzes, dem total adrenalektomierten Tiere die Möglichkeit, das weitere Leben zu befähigen, und in diesem Punkt ist ihre Wirkung dem Rindengewebe der Hauptnebenniere gleichsinnig, was von vielen Forschern, vor allem nach dem Experimente von Kojima[8] klar gestellt worden ist.

Die vorliegende Arbeit ist in erster Linie mit der androgenen Zone Grollman's nämlich der Fetalzone des Menschen und der X-Zone der Maus beschäftigt, wobei folgende Fragen gestellt sind.

1. Wie weit bestehen Ähnlichkeiten zwischen beiden Geweben?
2. Wie weit besitzen beide Gewebe Spezifität gegenüber den übrigen Rindenschichten?

Auf der anderen Seite sind die NN und die akz. NN am Kaninchen sowohl in normalen als auch in gewissen experimentellen Zuständen untersucht. Dabei erforscht man die histologische bzw. histochemische Beziehung zwischen beiden Geweben und die Möglichkeit des Bestehens einer region-bedingten Differenz der Beschaffenheiten unter den akz. Knötchen, die sich in verschiedenen Stellen der Bauch- bzw. Beckenhöhle befinden.

Beschreibung der Versuche

Material und Methodik

Zur Erforschung der X-Z verwandte ich 48 weisse Mäuse, u. zw. 20 Männchen im Alter von 18 bis 40 Tagen und 28 Weibchen von 18 bis 167 Tagen. Zum Vergleich wurden jüngere Tiere im Alter von 3 bis 15 Tagen benutzt. Zur Beobachtung der menschlichen Fetalzone standen

mir frische NN aus 19 Feten von 5 bis 10 Monaten zur Verfügung, die alle binnen 3 bis 10 Stunden nach Kaiserschnitt, Perforation, künstlicher Frühgeburt und künstlichem Abort zur Untersuchung gebracht wurden. Ferner wurden die NN sowie die akz. NN von 110 Kaninchen beiderlei Geschlechts mit dem Körpergewicht von 1230 bis 2750 g zur Berichtigung unterzogen. Für die Untersuchung der Fettsubstanzen dienten Sudan III, Nilblausulfat und die Methode nach Smith-Dietrich ; daneben wurde das Doppelbrechungsvermögen untersucht. Weiter wurden die Kon'sche Silberreaktion[9] u. zw. A-Methode nach diesem Autor und die Oxydasereaktion vorgenommen ; bei der letzteren wurde die Methode zur labilen Oxydase nach Katsunuma[10] angewandt. Bei der NN und akz. NN bei Kaninchen stellte ich ausserdem die Injektion von Diphtherietoxin sowie die Vitalfärbung mit Lithionkarmin zur Verfügung.

I. Befunde der NNR bei Mäusen und menschlichen Feten.

a. Fettsubstanz der NNR bei Mäusen.

Es ist schon von vielen Autoren wie Kolmer[11] u.a. klargemacht worden, dass Fettsubstanz der NNR bei Mäusen hauptsächlich in der Fasciculata und in den meisten Fällen ein wenig auch in der Glomerulosa nachweisbar ist. Meine Sudanpräparate bekunden die ähnlichen Bilder wie die bisherigen Schilderungen. Durch Nilblausulfat färbt sich die Fasciculata metachromatisch wie Löwenthal's Angabe.[12] Die Befunde der doppelbrechenden Substanz bieten nach Kawamura[13] starke individuelle Verschiedenheiten dar. Im Gegensatz zu Leffkowitz und Rosenberg[14] konnte ich diese Substanz in allen Fällen ausnahmslos nachweisen, wenn auch gewisse individuelle Schwankungen bestanden.

In der X-Z ist fast gar keine Fettsubstanz in jener grobtropfigen Form zu bemerken, wie sie sich in der Glomerulosa und Fasciculata nachweisen lässt, mit Ausnahme der jenigen weiblichen Tieren, bei denen die X-Z das Bild zeigt, das von den meisten Forschern als Rückbildungszeichen dieser Zone betrachtet worden ist. Die X-Z ist jedoch keineswegs durchaus frei von Fettsubstanz. Sie treten entweder diffus oder in Form ganz feiner Granula auf. Die erste Form äussert sich dadurch, dass bei Fettfärbung oft das Zytoplasma in der X-Z überall oder um den Kern herum diffus schwach angefärbt wird. Diese Färbung tritt oft in den inneren Lagen der X-Z intensiver in Erscheinung als in den äusseren. Gegen die Smith-Dietrich'sche Färbung und die Doppelbrechung verhält sie sich im allgemeinen negativ. In der zweiten Form, die häufig mit der ersten zusammen auftritt, sind die Fettgranula viel feiner als in der übrigen Rindenzonen. Ihre Verteilung ist sehr ungleichmässig und unregelmässig. Auch je nach der Zelle kann ihre Menge und Verteilung verschieden sein. In keinem Falle erreicht jedoch ihre Menge die in den übrigen Rindenschichten. Nach der Smith-Dietrich'schen Methode lassen sie sich, abgesehen

von den selten angetroffenen Ausnahmen, nicht färben. Die Doppelbrechung erweist sich im allgemeinen als negativ, nur selten ist sie in ganz beschränktem Masse positiv. Wir fanden die Fettsubstanz derartiger zwei Formen in der X-Z von 11 Tieren unter 20 Fällen.

b. Fettsubstanz der NNR bei menschlichen Feten.

Im 5. Fetalmonat sind feine sudanophile und Smith-Dietrich-positive Tröpfchen in allen Rinderteilen fast gleichmässig verbreitet. Auch die doppelbrechende Substanz verteilt sich in Form feiner Pulver fast überall zerstreut. Mit dem fortschreitenden Schwangerschaftsmonat wird die Fettsubstanz allmählich lokalisierend, indem die Tröpfchen in der äusseren Zone an Grösse und Menge zunchmen, der inneren hingegen an Menge abnehmen, abgesehen von den gröberen Tropfen in der direkten Umgebung der Markvene. Gegen Ende der Schwangerschaft nehmen sie in der inneren Zone wieder zu, aber ihre Grösse und Verteilung sind unregelmässig. Ähnliche Befunde erhält man in der Doppelbrechungsprobe und mit der Färbung nach Smith-Dietrich.

c. Kon'sche Silberreaktion der NNR bei Mäusen.

In der Glomerulosa und Fasciculata treten die feinen Silbergranula entweder gleichmässig auf, oder sie nehmen von des ersteren nach der letzteren hin allmählich an Menge ab, selten kommen sie reichlich in der inneren Partie der Fasciculata in Erscheinung. Die Granula sind meistens in Zytoplasma diffus, selten nur um den Kern herum verteilt.

In der X-Z sind bei beiden Geschlechtern die feinen hellfarbigen Granula dicht gedrängt und füllen das ganze Protoplasma aus.

d. Kon'sche Silberreaktion der NNR bei menschlichen Feten.

In der äusseren Rindenschicht kommt mässige Menge von feinen Granula diffus in Zytoplasma oder selten perinukleär vor. In der inneren Zone ist die Menge der Granula trotz zunehmender Zellgrösse verhältnismässig gering. Hier ist die Verteilung der Granula sehr ungleichmässig, so dass die granulahaltigen Zellen und die granulafreien vielfach durcheinander vermengt sind. Auch die Zellen mit schwarzen Granula und die mit brauen stehen nicht selten aneinander. Innerhalb der Zellen verteilen sich die Granula auch mannigfaltig, sie können nur im beschränken Teil der Zelleibs lokalisiert gefunden werden. Aus dem obigen Befunde ist anzunehmen, dass in der Fetalzone des Menschen deutliche Silbergranula festgestellt sind, die jedoch im Vergleich zu denen der äusseren Rindenzone trotz der Zunahme von Zellgrösse in relativ geringer Menge angetroffen und zugleich in sehr unregelmässiger Weise verteilt sind. Bezüglich der Schwangerschaftsmonate zeigen die Berunde der Silbergranula, im Rahmen dieser Beobachtung, keine merklichen Schwankungen.

e. Oxydasereaktion der NNR bei Mäusen und menchlichen Feten.

Über die Oxydasereaktion der NN finden wir die Beschreibungen von Gräff[15] und Katsunuma[10] in der Literatur.

In meiner Untersuchung kommen bei Mäusen, in der Mehrzahl der Fälle, die Oxydasegranula in der Glomerulosa sowie in inneren Teil der Fasciculata in geringer bzw. mittlerer Menge vor. In der X-Z fällt die Reaktion stets positiv aus u. zw. in stärkerem Masse wie den übrigen Rindenzonen. Am stärksten tritt sie in inneren Teil dieser Zone. In der Reaktion stets positiv aus u. zw. in stärkerem Masse wie in den übrigen degenerierenden X-Z wird die Reaktion abgeschwächt.

Was die Oxydasereaktion in der NN der meschlichen Feten anbelangt, so erweist sich in meiner Beobachtung die NN des 5 monatigen Fetus als oxydasenegativ. Im 6. Monat tritt eine diffuse bläuliche Färbung an der peripherischen Rindenschicht auf; in den folgenden Fetalmonaten wird die Reaktion immer stärker und von 8. Monat an fällt sie bisweilen auch in der inneren Rindenzone positiv aus, sie tritt aber ungleichmässig und nur in schwachem Grade auf.

II. Befunde der NNR und der akz. NN beim Kaninchen.

a. Struktur der akz. NN.

In der äusseren Schicht der akz. NN, die bei uns ausschliesslich aus Rindengewebe besteht, sind die Zellen bisweilen einigermassen knäuelartig angeordnet; zeitweise ordnen sie sich bandartig parallel mit der Kapsel an, oder sie können einfach dichte Anhäufung zeigen ohne besondere Anordnung zu nehmen. Im subkapsulären Gebiet sind die Parenchymzellen meistens klein, deren Cytoplasma bald hell, bald dunkel und schwach granuliert ist. Im inneren Teil des Knötchens sind die Zellen meistens plasmareicher und ordnen sich gewöhnlich in Strängen, die oft rädiar verlaufen und durch Blutkapillaren voneinander getrennt sind. Zuweilen können die Balken zellreich und mehr plump sein, oder die Zellen sammeln sich in einfache Haufen. Im inneren Teil haben die Zellen oft das Aussehen von Spongiozyten, aber sie können die Beschaffenheit der Zellen der Reticularis bzw. Juxtamedullarrinde zeigen. Manchmal entbehrt das Knötchen der Schicht der subkapsulären kleineren Zellen teilweise oder in ganzer Zirkumferenz.

Aus dem obigen ist es ersichtlich, dass die histologischen Bilder der akz. NN, im oben angeführten Sinne, keineswegs immer die gleichen sind. Diese strukturellen Schwankungen sind niemals an die Körperregion innig gebunden, wo die Knötchen sich befinden, so können z.B. zwei dicht aneinander liegenden Knötchen verschieden gebaut werden.

b. Fettsubstanz der NNR und der akz. NN.

Über die Fettsubstanz der NNR beim Kaninchen haben wir umfangreiche Mitteilungen, vor allem die ausführliche Beschreibung von Krylow[16]. Meine Befunde können im wesentlichen seine Angaben be-

stätigen. Die Färbung von Smith-Dietrich fällt in der Glomerulosa meistens negativ oder spurweise positiv aus. Nach Fasciculata hin verstärkt sich die Reaktion. Nach Sonoda[17] bleibt das genannte Verfahren in der Fasciculata negativ, während ich, wie Krylow und Sano[18] hier reichlich blaue Granula gefunden habe. In der Reticularis have ich gegen die Angabe nach Sano deutlich positive Reaktion festgestellt, wenn auch hier die Granula sich zumeist locker verteilen. In der juxtamedullären Rinde fällt die Reaktion häufig nur spurweise oder kaum positiv aus. Die doppelbrechende Substanz ist in der Glomerulosa entweder gar nicht oder nur spurweise, in der Fasciculata reichlich und fast gleichmässig oder in Haufen gefunden. In der Reticularis ist sie im Gegensatz zu Krylow deutlich nachzuweisen wie in den Angaben von Sonoda und Sano. Die Menge ist weniger als in der Fasciculata und nach der Tiefe zu nimmt sie ab. In der juxtamedullären Rinde ist sie sehr wenig oder zuweilen nicht nachweisbar. Das doppelbrechende Vermögen verschwindet durch Erwärmung und kommt durch Abkühlung wieder zum Vorschein, wobei die typische Kreuzfigur wahrzunehmen ist.

Was die Fettsubstanz in der akz. NN beim Kaninchen anbelangt so finden wir die Berichte von Mulon und Porak[19]. In meiner Beobachtung tritt in der subkapsulären Lage des Knötchens die Fettsubstanz, wenn auch in geringer Menge, in der Mehrzahl der Fälle auf. Im Zelleib findet sich diese Substanz zumeist nur im beschränkten Bezirk, vornehmlich auf derjenigen Seite der Zelle, die der fibrösen Kapsel des Knötchens oder den Blutkapillaren zugewandt ist, welch letzlere zwischen den Zellsträngen verlaufen. Derartige Lokalisation der Fettsubstanz ähnelt derjenigen in der Glomerulosa der Hauptdrüse. Im zentralen Teil des Knötchens zeigt sich der Fettgehalt selten in gleichem Masse wie in der Fasciculata der Hauptdrüse sondern sie kommt in den meisten Fällen weniger vor. Die Verteilung ist hier häufig unregelmässig. Die Doppelbrechung und die Färbung nach Smith-Dietrich fällt positiv aus u. zw. im allgemeinen fast in desselben Verteilung wie die sudanophile Substanz. Allgemein gesagt, kommt beim Kaninchen die Fettsubstanz in der akz. NN oftmals weniger als in der Rinde des Hauptorgans vor. Aber es besteht eine grosse Schwankung, so dass selbst unter denjenigen Rindenknötchen, die in ein und derselben Körperregion eines Tieres dicht aneinander liegen, eine ausgesprochene Differenz an Fettgehalt konstatiert werden kann. Auch ein Knötchen durch Bindegewebszüge in mehrere Zellhaufen eingeteilt werden, die untereinander verschiedenen Fettgehalt zeigen. Dem Fundort des Knötchens nach bemerkt man niemals eindeutigen Unterschied im Verhalten der Fettsubstanz.

c. Fettsubstanz der NNR nach einseitiger Suprarenalektomie und der akz. NN nach totaler Suprarenalektomie.

Es ist eine bekannte Tatsache, dass die Fettsubstanz der NNR nach einseitiger Suprarenalektomie abnimmt. In meiner Beobachtung an dem bleibenden Nebennieren der Kaninchen, die 10 bis 52 Tage nach einseitiger Suprarenalektomie überlebten, erhält man ähnliche Resultate. Die akz. NN, die 18 bis 57 Tage nach doppelseitiger Suprarenalektomie bzw. 30 bis 98 Tage nach einseitiger Nebennierenexstirpation untersucht wurden, zeigen ähnliche Veränderungen wie das Hauptorgan auf, welches nach einseitigen Exstirpation zur Beobachtung kommt. Die Zellbalken sind oftmals plump. Mitosen werden häufig angetroffen. Sie kommen hauptsächlich aber keineswegs immer in der äusseren Schicht des Knötchens vor. Die Fettsubstanz tritt häufig in ganz geringer Menge verstreut oder lokalisiert in Erscheinung. Ihre Abnhame tritt viel deutlicher auf als in der hypertrophischen Rinde der bleibenden NN nach einseitiger Exstirpation.

d. Kon'sche Silberreaktion der NNR und der akz. NN.

Über die Kon'sche Reaktion der NN beim normalen Kaninchen gibt es eingehende Veröffentlichungen aus Kon'schen Institut und meine Resultate decken sich im grossen und ganzen mit ihnen. Die Befunde sind indessen ziemlich grossen individuellen Schwankungen unterworfen. Über diese Reaktion der akz. NN haben wir in der Literatur nur eine einzige kurze Belehrung in der Arbeit von Sonoda[17]. In meiner Untersuchung sind feine Silbergranula in der äusseren Schicht zumeist diffus im Zytoplasma, bisweilen aber nur in der Umgebung des Kerns gefunden. Im Zentralteil des Knötchens fällt die Reaktion oftmals in ähnlichen Weise, nicht selten aber sehr schwach oder fast negativ aus. In der akz. NN stellt sich hin und wieder die Reaktion je nach der Zelle sehr verschieden stark ein, was zwar im Hauptorgan zuweilen bemerkbar, aber hier viel ausgesprochener zu Tage tritt; und nicht selten findet man die stark positiv reagierende Zelle neben der fast negativen.

In der hypertrophischen Rinde des NN, die 18 bis 48 Tage lang nach einseitiger Suprarenalektomie in Körper zurückgeblieben waren, fällt die Kon'sche Reaktion etwas stärker aus, was mit dem Ergebnis von Sonoda übereinstimmt.

In den akz. NN, die 14 bis 62 Tage nach doppelseitiger Suprarenalektomie, d.h. 39 bis 83 Tage nach einseitiger Nebennierenexstirpation zur Untersuchung kamen, zeigen die Resultate im grossen und ganzen keine merklichen Unterschiede gegenüber denjenigen in Normaltieren oder zuweilen eher leichte Abschwächung der Reaktion. Dabei muss man die Existenz eines ziemlich grossen individuellen Schwankung in Erwägung ziehen.

e. Oxydasereaktion der NNR und der akz. NN.

Die Oxydasegranula sind in der Glomerulosa in der meisten Fällen

nachweisbar, u.zw. in kleiner Menge und ungleichmässigen Verteilung. In der Fasciculata fehlen sie zumeist, abgesehen von denjenigen Fällen, in denen diese Zone wenig Fettsubstanz trägt. In der Reticularis und in der juxtamedullären Rinde fällt die Reaktion stets deutlich positiv aus.

In der akz. NN sind die Oxydasegranula fast immer zu konstatieren, u.z.w. bald überall gleichmässig, bald ungleichmässig in beschränkter Weise. Es bestehen in Verhalten dieser Reaktion keine eindeutigen Verschiedenheiten zwischen der Knötchen in der Nähe des Hauptorgans und denen, die vom letzteren entfernt, wie in die Gegend der inneren Genitalien, gefunden werden.

In den NN, die 21 bis 31 Tage lang nach einseitiger Exstirpation im Körper geblieben waren, bemerkt man eine geringe Zunahme an Stärke der Reaktion.

Die akz. NN, die 55 bis 76 Tage nach doppelseitiger Adrenalektomie, bzw. 76 bis 97 Tage nach einseitiger Exstirpation zur Untersuchung gebracht worden sind, zeigen eine deutliche Zunahme der Oxydasegranula, obwohl sie in der Verteilung mehr oder weniger unregelmässig auftreten.

Es wurden die nach dem 3 bis 8 Tage dauernden Hungerzustand getöteten Tiere zur Untersuchung der Oxydasereaktion herangezogen. In der Mehrzahl der Fälle tritt eine Verstärkung der Reaktion ein, was zwar in allen Rindenzonen, aber besonders auffallend in der Glomerulosa und Fasciculata geschieht. Auch in den akz. NN wird die Reaktion intensiver.

f. Veränderungen der NNR und der akz. NN nach Diphtherietoxininjektion beim Kaninchen.

Die minimale Letaldosis der gebrauchten Toxin beträgt für ein Meerschweinchen von Körpergewicht 250 g. $\frac{1}{1000}$ cc. 12 bzw. 64 Stunden nach subkutaner Einführung dieser Toxin in Mengen von 0.002 bis 0.03 cc. wurden die Versuchstiere geschlachtet.

Das von mir erhaltene Resultat der NNR stimmen in den grossen Zügen mit den Angaben früherer Autoren überein. In der akz. NN kann man die gleichen Veränderungen finden wie im Hauptorgan. Nämlich ausser den Zirkulationsstörungen werden verschiedene regressive Veränderungen fetgestellt. Sie erreichen jedoch nur selten das Mass in Hauptorgan, zumal so auffallende Nekrose wie im letzteren ist nirgend aufzufinden. Aber die Intensität dieser Veränderungen ist nicht immer gleich unter den Knötchen, die sich in derselben Körperregion eines Tieres nebeneinander vorfinden.

g. Vitalfärbung der NNR sowie der akz. NN mit Lithionkarmin.

4%ige Lithionkarminlösung wurde mit dem Intervall von 24 Stunden intravenös injiziert. Die gesamte Menge beträgte 80 bis 135 cc. Die

Verrsuchstiere wurden 24 Stunden nach der letzten Injektion getötet.

Wie in der Berichte von Kiyono[20] treten in meiner Beobachtung feine Karmingranula im Protoplasma der Rindenzellen vor. Meistens sind sie in der äusseren Zone der Fasciculata am deutlichsten erkennbar. Selbst in den nebeneinander stehenden Drüsenzellen zeigen sich die Farbstoffgranula nicht immer in gleicher Deutlichkeit. Ferner sind gröbere Karmingranula in den Endothelien und Histiozyten nachweisbar genau wie in der Beschreibung der bisherigen Autoren.

Die akzessorischen Rindenknötchen, die von mir zu diesem Zweck untersucht wurden, waren alle nahe der Keimdrüse gelegt. Die Karmingranula erscheinen in der Parenchymzellen wie beim Hauptorgan. An der Peripherie des Knötchens kommen die Karmingranula im allgemeinen schwerer zum Vorschein wie in der Glomerulosa der Hauptdrüse. In allen Knötchen, die nebeneinander gelegt sind, ist das Bild der Vitalfärbung nicht immer das gleiche.

Besprechung

Über die Fettsubstanz in der X-Z der Mäusenebenniere, in der noch keine regressiven Veränderungen zu sehen sind, geben die Meinungen der Autoren auseinander. Während eine Reihe von Autoren diese Substanz konstant bemerkten[21][22][23][24], wiesen die anderen hier keinerlei Fettsubstanz nach[1][25]. Nach Deanesly[26] kommt beim Weibchen das Lipoid in der wachsenden X-Z vor.

Wie oben angeführt steht so fest in meiner Beobachtung, dass die X-Z, wo noch keine Degeneration auftritt, sich gegen Sudan III oft ganz negativ verhält, aber dass nicht selten eine diffuse oder feingranuläre Färbung der Drüsenzellen zeigt. Diese Fettsubstanz nimmt bei Nilblausulfatfärbung etwas mehr blaue Farbennuance auf, wie die in den übrigen Rindenschichten. Das Smith-Dietrich'sche Verfahren sowie der Doppelbrechungsversuch fallen in dieser Zone nur selten stellenweise schwach positiv, im allgemeinen jedoch negativ aus.

Obwohl die Involution in der NNR des Menschen bekanntermassen augenfälligst nach der Geburt erscheint, kann sie schon intrauterin auftreten[27]. Ich sah diese Veränderung bei 8 monatigen Feten ganz deutlich im tieferen Teil der Fetalzone.

Was die Fettsubstanz in der NNR der menschlichen Feten anbelangt, so gibt es in der einschlägigen Literatur als allgemein anerkannt, dass etwa in der zweiten Hälfte der Fetalzeit die Fettsubstanz in der äusseren Zone deutlich nachweisbar ist. Auch in der Drüsenzellen der inneren Zone kommt sie vor, die aber nach vielen Autoren derjenigen in der ersteren an Menge nachsteht.

Gegenüber den Angaben von Elliott und Armour[28] und Grollman[3]

have ich die änisotrope Substanz nicht nur in der äusseren Zone sondern auch in der inneren, wenngleich in weniger Menge, in klarer Weise festgestellt. Es gilt also nicht als genug gerechtfertigt zu sein, das Fehlen der anisotropen Substanz als charakteristisch für die Fetalzone anzusehen. Es fällt mir auf, dass die Fettröpfchen in der inneren Rindenschicht oft feiner sind und bei Nilblausulfatfärbung mehr blauen Farbenton aufnehmen in Vergleich zu denen in der äusseren Schicht.

Wenn man die Befunde der Fettsubstanz, die in der X-Z ohne erkennbare Degeneration bei Mäusen gewonnen sind, mit denen der Innen- bzw. Fetalzone der NNR bis menschlichen Feten miteinander vergleicht, so kann man kurzweg folgendermassen darlegen.

Als mehr oder weniger ähnlich zu betrachten:

1. In der Fetalzone kommt die Fettsubstanz weniger vor als in der äusseren Zone. In der X-Z fehlt sie gänzlich, oder ist weniger nachweisbar als in den übrigen Rindenzonen.

2. Die in der menschlichen Fetalzone vorkommende Fettsubstanz ist hauptsächlich fein granulär. Die zeitweise in der X-Z auftretende Fettgranula sind auch sehr fein.

3. Die Fettgranula in der Fetalzone äussern sich bei Nilblausulfatfärbung etwas mehr blau als in der äusseren Zone. Die in der X-Z ohne erkennbare Degeneration vorkommende Fettsubstanz färbt sich auch etwas mehr blau als in den anderen Rindenzonen.

Als widersprechend zu betrachten:

1. In der Fetalzone tritt die Fettsubstanz viel häufiger und deutlicher in Erscheinung als in der X-Z. In der letzteren kommt sie hingegen inkonstant und nur spärlich zutage.

2. Die doppelbrechende Substanz ist in der Fetalzone stets nachweisbar, während sie in der X-Z nur selten schwach positiv zu finden ist.

3. Das Verfahren nach Smith-Dietrich ergibt sich in der Fetalzone als positiv, während dasselbe in der X-Z selten positiv ausfällt.

Es ist mir augenblicklich schwer zu beurteilen, wie viel von der Fettsubstanz in der Fetalzone als von degenerativer Natur vom eigentlichen Bestandteil zu unterscheiden.

Jedenfalls geht aus meiner obigen Erörterung hervor, dass die Annahme von Grollman, Fetalzone und X-Z als homologe Gewebe von übrigen Rindengeweben sonderzustellen, wenigstens insofern als schwach begründet zu beurteilen ist, als diese Auffassung sich teils auf den histochemischen Befunden der Fettsubstanz zu stützen scheint.

Über die Kon'sche Silberreaktion der NNR bei Mäusen findet sich eine kurze Beschreibung von Sonoda[17]. Maruyama[29] nahm diese Reaktion an einem menschlichen Neugeborenen vor, die im Mark schwach positiv, in der Rinde dagegen negativ ausfiel. Wenn man in meiner Be-

obachtung die Befunde an der X-Z mit denen an der Fetalzone miteinander vergleicht, so ist folgendes zu erheben. In der X-Z sind in der Mehrzahl der Fälle die Silbergranula bemerkbar, welche in der dichteren Verteilung im Zelleib und in der helleren Farbe einigermassen von denen der übrigen Zonen zu unterscheiden sind. In der Fetalzone lassen sich diese Granula in der Verteilung und Menge gewisse Unterschiede gegenüber denen der äusseren Zone erkennen, was aber wenigstens zum Teil mit dem Degenerationsprozess dieser Zone in Zusammenhang zu stehen scheint. Jedenfalls können wir keine Ähnlichkeiten im Bild der Silbergranula zwischen Fetalzone und X-Z feststellen.

Wenn man die Oxydasereaktion der X-Z mit derjenigen der Fetalzone vergleicht, so fällt sie bei der ersteren stark positiv aus, während sie bei der letzteren allein in späteren Fetalmonaten und inkonstant und schwach eintritt. Also in Bezug auf das Verhalten gegenüber der Oxydasereaktion bestehen gewisse Verschiedenheiten zwischen beiden Geweben.

Was die akz. NN bei Kaninchen anbelangt, so ist in ersten Linie die grosse Schwankung in ihren histologischen Bildern hervorzuheben. Allgemein gesagt, entspricht die akz. NN selbstverständlich strukturell wenigstens einem Teil der Rindenschicht des Hauptorgans ; aber, wie Aichel und Pick erörtern, kann das Bild je nach dem Knötchen sehr schwanken, und selbst nebeneinander stehende Knötchen zeigen eher selten die gleiche Struktur bzw. Zellbeschaffenheit. Dasselbe gilt von der Fettsubstanz, Kon'scher Silberreaktion und der Oxydasereaktion der akz. NN.

Immerhin gehen die Ähnlichkeiten in Bezug auf histochemische Befunde zwischen der Fetalzone des Menschen bzw. der X-Z der Maus einerseits und der Marchand'schen NN des Kaninchens andereseits keineswegs so weit, dass man diesen Geweben als eine besondere Gruppe einen Sonderplatz gegenüber den übrigen Rindengeweben bzw. den anderen, besonders in der direkten Nähe des Hauptorgans liegenden akz. Knötchen zugeben kann.

Beim Kaninchen sowohl in der nach einseitiger Suprarenalektomie bleibenden NN als auch in der akz. NN nach doppelseitiger Suprarenalektomie ist eine Verminderung der Fettsubstanz nachweisbar, zumal deutlich in der letzteren. Unter derartiger Bedingung zeigt die Kon'sche Silberreaktion leichte Verstärkung in der Hauptdrüse, während sie in der akz. NN, wenn auch ziemlich inkonstant, fast in gleichem Mass oder etwas schwächer wie in der Norm vor sich geht. Die Oxydasereaktion scheint in der zurückgebliebenen NN ein wenig verstärkt zu sein ; in der akz. NN nimmt sie nach Exstirpation beider Hauptdrüsen deutlich an Intensität zu. Im Hungerzustand nimmt die Fettsubstanz wesentlich sowohl im Hauptorgan als auch in der akz NN ab, während sich die Oxydasereaktion im umgekehrten Sinne verändert.

Durch Injektion von Diphtherietoxin konstatierte ich bei Kaninchen die Veränderungen, die von den bisherigen Autoren beschrieben worden waren, nicht nur in der Hauptnebenniere sondern auch in der akz. NN. Die Alterationen in der letzteren treten nicht immer in gleichen Grad, oft schwächer ein wie im Hauptorgan. Dieser Umstand ist verständlich, wenn man bedenkt, dass auch im Hauptorgan die Veränderung nie durchweg in gleicher Art zustande kommt. Hier ist auch zu erinnern an die Schilderung von Marchetti[30], nach welcher die akz. NN als Reserveorgan zu betrachten sei.

Zusammenfassung

1. Es wurden in der Rinde der Nebenniere bei menschlichen Feten und Mäusen die Untersuchungen von Fettsubstanz, Oxydasereaktion sowie Kon'scher Silberreaktion vorgenommen, wobei von allem die Befunde der Fetalzone bei Menschen und die der X-Zone bei Mäusen miteinander, sowie mit denen der anderen Rindenzonen in Vergleich gesetzt wurden.

2. In Bezug auf die Befunde der Fettsubstanz, Kon'scher Silberreaktion und der Oxydasereaktion bestehen zwischen der X-Z und den anderen Rindenzonen der Mäusenebenniere gewisse Unterschiede. Die Fetalzone der menschlichen NN lässt sich ebenfalls hinsichtlich der obigen histochemischen Befunde mehr oder weniger deutliche Verschiedenheiten gegenüber den anderen Rindenzonen erkennen.

3. Durch unsere histochemischen Untersuchungen ist festgestellt, dass zwischen der Fetalzone der Menschen und der X-Z der Maus, zwar einerseits gewisse Ähnlichkeiten, andererseits aber viele Verschiedenheiten existieren. Demnach zeigt sich, dass vom histochemischen Standpunkt aus die hypothetische Annahme, beide Gewebe zusammen als einheitliches „androgenes Gewebe" zu betrachten und vom übrigen Rindengewebe sonderzustellen, nicht ausreichend im positiven Sinne bewiesen ist.

4. An der NN und der akz. NN von Kaninchen wurden histologische Untersuchungen sowie Beobachtungen über das Verhalten gegenüber der Vitalfärbung und der Schädigung mit Diphtherietoxin ausgeführt.

5. Die strukturellen und histochemischen Beschaffenheiten der akz. NN bei Kaninchen im normalen und pathologischen Zustand ähneln selbstverständlich denen des Hauptorgans, weisen jedoch untereinander ziemlich grosse Schwankungen auf, welche keineswegs durch die Körperregion bedingt sind, wo die Knötchen sich befinden. Daraus erhellt, dass sich die Annahme, nach welcher die in der Keimdrüsengegend vorhandene akz. NN allein als von androgener Natur von der in direkter Nähe des Hauptorgans befindlichen sonderzustellen sei, in keiner Weise mit den Ergebnissen meiner Untersuchungen stützen lässt.

Literatur

1) Howard-Miller, Am. J. Anat., 1927, **34**, 909.
2) Waring, Quart. J. micr. Sci., 1936, **78**, 329.
3) Grollman, The adrenals, Baltimore, 1936.
4) Vines, The Adrenal Cortex and Intersexuality, London, 1938.
5) Aichel, Arch. f. mikr. Anat. u. Entw., 1900, **56**, 1.
6) Pick, Arch. f. Gyn., 1901, **64**, 670.
7) Poll, Arch. f. mikr. Anat. u. Entw., 1903, **62**, 138.
8) Kojima, Tohoku J. Exp. Med., 1929, **13**, 203.
9) Kon, Silberreaktion u. Histochemie der endokrinen Organe (jap.), Tokio, 1938.
10) Katsunuma, Intracelluläre Oxydation u. Indophenolsynthese, Jena, 1924.
11) Kolmer, Arch. f. mikr. Anat., 1918, **91**, 1.
12) Löwenthal, Anat. u. Path. d. Spontanerkrank. d. kl. Laboratoriumstiere (Jaffe), Berlin, 1931, 363.
13) Kawamura, Die Cholesterinesterverfettung, Jena, 1911.
14) Leffkowitz u. Rosenberg, Frankf. Z. Path., 1926, **34**, 174.
15) Gräff, Frankf. Z. Path., 1912, **11**, 358.
16) Krylow, Ziegler's Beitr. pathol., 1914, **58**, 449.
17) Sonoda, Hokkaido Igaku Zassi, 1929, **7**, 1570.
18) Sano, J. Endocrinol. u. exp. Therap (jap.), 1935, **4**, 177.
19) Mulon u. Porak, C. R. Soc. Biol., 1913, **75**, 258 u. 313.
20) Kiyono, Heutiger Stand d. Vitalfärbung u. ihrer Untersuchungsmethode, Tokio, 1921.
21) Masui u. Tamura, Jap. J. Zo. Sci., 1924, **1**, 55.
22) Hett, Z. mikr.-anat. Forsch., 1928, **13**, 428.
23) Kukita, Jap. J. Exp. Med., 1930, **14**, 38.
24) Inohara, Nippon Fujinka Zassi (jap.), 1933, **28**, 1431.
25) Nürnberger, Z. mikr.-anat. Forsch., 1932, **28**, 589.
26) Deanesly, Proc. Roy. Soc. B., 1928, **103**, 523.
27) Keene u. Hewer, J. Anat., 1926–27, **61**, 602.
28) Elliott u. Armour, J. Path. a. Bact., 1911, **15**, 481.
29) Maruyama, Hokkaido Igaku Zassi (jap.), 1938, **16**, 1887.
30) Marchetti, Virchow's Arch. Path., 1904, **177**, 227.

The Tohoku Journal of Experimental Medicine, Vol. 69, Nos. 2-3, 1959

Studies on Cirrhosis of the Liver

V Etiology

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(Received for publication, January 14, 1959)

Introduction

Cirrhosis of the liver is a very important disease forming the nucleus of various liver diseases. In Japan it has temporarily dropped in frequency during World War II, but in recent years it has become more frequent again. Studies on this disease have become active in these years and marked progress has been made in the field of its pathophysiology and diagnostic technics, but on its etiology we are as yet in the dark on many points. It is known that alcohol has been cited as the chief causative factor of liver cirrhosis and more recently viral hepatitis has received much attention concerning the etiology of cirrhosis, but there are as yet many questionable points in the matter. In Japan, there are as yet controversies concerning the significance of alcohol and viral hepatitis as the cause of liver cirrhosis. We have already described our study on the pathogenesis of liver cirrhosis based on animal experiments, and in this article, we will report on the same subject as studied from the angle of clinical observations.

Clinical Findings

Subjects

Our subjects of study consisted of 61 liver cirrhosis cases accommodated in our clinic in the period of 1920–57, comprising 23 pre-war cases treated from 1920 to 1943 and 38 post-war cases treated from 1946 to 1957.

Findings and Discussion

1. Frequency by sex and age

The sex ratio of liver cirrhosis cases was 6.7:1 and 4.7:1 among the pre-war and post-war cases, respectively, or ca. 5:1 in the total of our subjects, the male cases widely predominating over the female cases.

The age of the first occurrence ranged between 7 and 69 years. Patients of ages from 41 to 60 years were here the most frequent among both the pre-war and the post-war cases, accounting for 59% of the total cases, while cases of 21 to 40 years represented the rather high frequency of 34%. The mean age of the first onset was 44.8 years among the pre-war and 42.1 among the post-war cases, or 43.1 years among the total subjects.

II. Pathogenesis

1) Relation to alcohol

In estimating the effect of alcoholic drinks in causing liver cirrhosis, the problem of the quantity of alcohol consumption large enough to justify considering it as the cause of the disease presents itself at first. Our survey on the relation between the hepatic changes and the liquor and food consumption of 18 drinkers who visited our clinic with complaints other than liver diseases, as shown in Fig. 1 and partially reported already,[3]

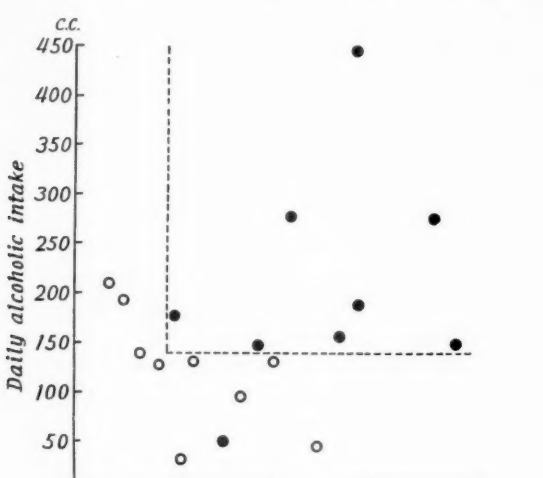

Fig. 1. Relation between alcoholic intake, duration of alcoholic intake, and histologic changes in alcoholics. ● Cases showing fatty metamorphosis and/or fibrosis. ○ Cases showing neither fatty metamorphosis nor fibrosis.

showed that 8 of 9 cases having fatty metamorphosis or fibrosis of the liver were those who recently had been drinking more than 140 cc in pure alcohol equivalent, taking less than 55 g of protein per day and having a history of alcoholic intake for more than 10 years, while the cases free of such hepatic changes were those who did not come up to this standard

of heavy drinkers. Histologic changes (fatty metamorphosis or fibrosis) were more frequent in the drinkers of above 140 cc in daily average than in those of less than 140 cc, and more frequent in those of more than 10 years of experience as drinkers than in those of less than 10 years of experience, the difference being stochastically significant in both cases.

We found among the 60 liver cirrhosis cases bibbers of more than 140 cc in pure alcohol equivalent in daily average in a stochastically significant frequency while such heavy drinker could not be found among the controls.

Accordingly, we felt justified in assuming that alcohol, when taken at the rate of 140 cc on the average per day at least recently and for more than 10 consecutive years, can cause liver cirrhosis. Of course, this criterion is based on our investigation of only 18 cases of heavy drinkers, and if we consider the difficulty of obtaining accurate information on the quantity of daily intake and the duration of the drinking habit and the individual differences in the ménus accompanying the drinking, such a criterion cannot be of absolute validity, but in comparison with the criteria proposed by other authors, we feel we have succeeded in laying down a more rational criterion based upon explicit grounds.

According to this criterion, we found such cases passing above the criterion in 27% of the pre-war and 13% of the post-war patients. (Tab. I).

TABLE I

Alcoholic Intake of Patients with Liver Cirrhosis and Control

	Before 1945		After 1945		Total	
	Liver cirrhosis (22 cases)	Control (22 cases)	Liver cirrhosis (38 cases)	Control (38 cases)	Liver cirrhosis (60 cases)	Control (60 cases)
Abstinence	36.3%	68.0%	36.8%	52.6%	36.7%	58.3%
Less than 54 cc daily	9.1	18.2	21.0	29.0	16.6	25.0
54–140 cc daily	27.3	13.8	29.0	18.4	28.3	16.7
More than 140 cc daily	27.3	0	13.2	0	18.4	0

2) Relation to viral hepatitis

Anamnesis of hepatitis was found in only 9% of the pre-war cases but in 29% of the post-war cases with liver cirrhosis, the difference from 0% of the control cases being clearly significant. The difference in the frequency between the pre-war and the post-war cases may perhaps reflect the difference by time in the consideration paid to hepatitis as a possible cause of liver cirrhosis, but it is also possible that, as Kalk has pointed out, the causes of liver cirrhosis may shift in relative frequency with age.

The time elapsing between the occurrence of hepatitis and that of

liver cirrhosis in our 13 cases ranged between 1½ months at the shortest and 7 years at the longest, and in the majority of cases—69%, between 2 and 6 years.

The interesting point in the relation between hepatitis and liver cirrhosis is the question of age of the first outbreak of hepatitis. Hepatitis is said to occur most frequently in children and youths, but the mean age of the 13 cases of liver cirrhosis caused by hepatitis was no less than 39.3 years and 92% of them had hepatitis first after 30 years. Generally speaking, however, liver cirrhosis patients are of mature age, and considering the possibility of some of them having forgotten their experience of hepatitis in childhood, we made the following survey. Two groups of controls of the same age constitution as that of the hepatitis-liver cirrhosis cases were examined in comparison ; in one of the groups, the frequency of past anamnesis of hepatitis which occurred at ages above 30 was 13%, while in the other, the frequency amounted to 54% (Fig. 2). Both these rates were significantly different from that in the cirrhosis cases. That is to say, the

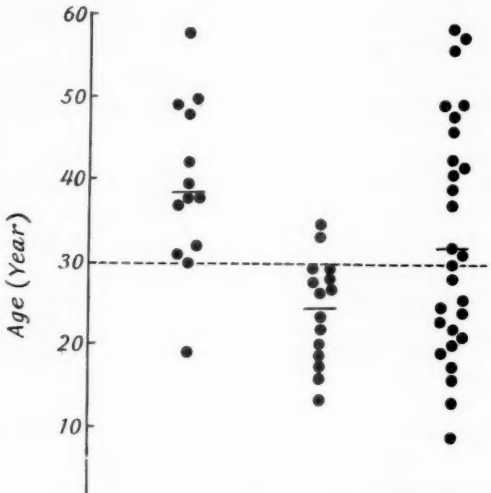

Fig. 2. The ages when patients with liver cirrhosis and controls had suffered from viral hepatitis.

age of the first occrrence of hepatitis is shifted to the high-age side in the cases who had subsequent liver cirrhosis. This fact also suggests the inverse relation that its development into cirrhosis of the liver is facilitated when viral hepatitis does occur in an age higher than 30 years. The observation by Mannbeck[5] and Jorke & Hanusch[6] that the cases who have contracted viral hepatitis in mature age suffer longer and more severely from it than the younger cases seems to support this opinion of ours.

3) Obesity and liver cirrhosis

Obesity could be presumed to have been the cause of liver cirrhosis in 2 (5%) of the post-war cases.

4) Analysis of 61 cases of liver cirrhosis by causes

With reference to the above criterion of heavy drinking and anamnesis of hepatitis, as well as histological findings obtained by liver biopsy, we have classified the 61 cases of liver cirrhosis by cause as shown in Tab. II. Among the pre-war cases, heavy alcohol consumption and malnutrition

TABLE II

Etiology of Liver Cirrhosis

Etiology	Before 1945	After 1945	Total
Alcohol and/or malnutrition	5 (21.7%)	5 (13.2%)	10 (16.4%)
Alcohol and viral hepatitis	2 (8.7%)	2 (5.2%)	4 (6.6%)
Viral hepatitis	0	9 (23.7%)	9 (14.7%)
Obesity	0	2 (5.2%)	2 (3.3%)
Unknown	16 (69.6%)	20 (52.7%)	36 (59.0%)

accounted for 21.7% and overdrinking plus hepatitis for 8.7%, while in the remaining 69.6%, the cause could not be ascertained. Among the post-war cases, hepatitis was the most frequent cause (in 23.7%), overdrinking and malnutrition (13.2%), overdrinking plus hepatitis (5%) and obesity (5.2%) following in that order, the cause being unknown in 52.7% of the cases.

Kalk reports that alcohol topped the list of causes of liver cirrhosis between 1931 and 1940, but in the statistics for 1950 and downwards, hepatitis has usurped the position, and he remarks on the alteration of the causes of the disease by age.[4]

In our study, we found a lower frequency of liver cirrhosis due to alcoholic intake and a higher frequency of those following hepatitis in the post-war patients. We believe such results are attributable to the prevalence of hepatitis and the forced restraint of drinking due to the deterioration of economics during and after World War II.

Thus, a change in the list of causes of liver cirrhosis by age has been

ascertained in our study too, but the recent increase in the consumption of alcoholic drinks accompanying the amelioration in the economic situation in Japan probably may involve a recovery of the significance of alcohol as a cause of liver cirrhosis in the near future. In fact, the alcohol consumption per capita in 1955 in Japan has risen 15% above that in 1935.

In several cases, both pre- and post-war, alcohol and hepatitis were found acting in combination in causing liver cirrhosis. By the liver biopsy findings applied to the post-war cases alone, we could observe pathohistological changes apparently suggesting that hepatitis was the primary cause of cirrhosis in these cases and leading to the inference that alcohol had expedited the development from hepatitis to liver cirrhosis.

In 2 of our post-war cases, obesity was assumed as the cause of the disease. Fatty liver is rather frequent among obese subjects, and fatty liver, when persistent, can lead to fibrosis and thence to cirrhosis, quite as in the case with heavy drinkers. Obesity should be noted as a possible cause of liver cirrhosis.

The cause of the disease is unknown in 69.6% of the pre-war and 52.7% of the post-war cases. What relation can be found between these cases and non-icteric hepatitis?

Non-icteric hepatitis, being rather frequent but showing only mild clinical symptoms, is said to be overlooked very often by the patients themselves and thus may escape medical attention. Therefore, we might consider this type of hepatitis as the cause lying behind some of the liver cirrhosis cases of "unknown cause". As this disease is so difficult to diagnose accurately, we cannot obtain any data assurring us of the pathogenicity of non-icteric hepatitis, and this seems an important theme awaiting future scrutiny.

5) Sex ratio by causes

The sex ratio (male: female) of the liver cirrhosis cases caused by overdrinking was 9:1, and that of the cases due to heavy drinking plus hepatitis 4:0, but the ratio was much smaller in the cases attributable to other causes, standing at 2:1 in the cases due to hepatitis alone and 1:1 in the cases of obesity. The ratio was 6:1 among the cases of unknown cause, standing between that of alcoholic and hepatitis cases.

6) Causes of the disease and age

The mean age in the groups of our subjects divided by causes of the disease (if we count out a case with infantile liver cirrhosis of 7 years) was 46.7 years in the group of the cases due to drinking and malnutrition and equally 46.7 years in the hepatitis-cirrhosis group, no difference by cause in this respect, as reported by Sherlock[7] and Baggenstoss,[8] being observable in our cases. In the cases due to drinking plus hepatitis, the mean age was 35.2 years, in the cases due to obesity, 34.5

years, and in the cases of unknown cause, 44 years.

Summary and Conclusion

Upon etiological study of 61 cases of liver cirrhosis treated at this clinic between 1920 and 1957, the present authors obtained the following results.

1. As the criterion of alcoholism as a cause of liver cirrhosis, the line of mean recent daily consumption of 140 cc of pure alcohol equivalent and duration of alcoholic intake for at least 10 years has been laid down.

2. In the development from hepatitis to cirrhosis the influence of age factor was pointed out, while in the cases of obesity followed by liver cirrhosis, the frequency of fatty liver in obese subjects suggested the possibility of development of fatty liver into cirrhosis.

3. Our etiological study based on the above criterion of overdrinking, anamnesis of hepatitis, and histological examination led to the findings that among the pre-war and wartime cases (treated before 1945), heavy drinking was the cause in 22% and drinking plus hepatitis in 9%, while the cause was not ascertainable in the remaining 69%; in the post-war cases, hepatitis was the most frequently ascertained cause (24%), while the percentage of overdrinking dropped to 13%; 5% each of the cases were due to overdrinking plus hepatitis and obesity, the remaining 52.7% cases revealing no ascertainable cause.

References

1) Nakamura, S., Tohoku J. Exp. Med., 1957, **65**, 241.

2) Nakamura, T., Nakamura, S., Sugawara, Katakura, Isono, Suzuki, Kaneko & Takizawa, *ibid.*, 1958, **67**, 105.

3) Nakamura, T., Nakamura, S., Kawamura, Kaneko, Sugawara, Katakura, Isono & Suzuki, *ibid.*, 1958, **67**, 365.

4) Kalk, Cirrhose und Narbenleber, Ferdinand Enke, Stuttgart, 1954.

5) Mannbeck, Acta Med. Scand., 1950, **47**, 443.

6) Jorke & Hanusch, Ärztl. Wschr., 1957, **12**, 666.

7) Sherlock, Diseases of the liver and biliary system, Blackwell, Oxford, 1955.

8) Baggenstoss & Stauffer, Gastroenterology, 1952, **22**, 157.

The Tohoku Journal of Experimental Medicine, Vol. 69, Nos. 2-3, 1959

Studies on Cirrhosis of the Liver

VI Treatment and Prognosis

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(Received for publication, January 14, 1959)

Introduction

In the previous paper,[1] the present authors have reported on the results of their study on the pathogenesis of liver cirrhosis.

We will report here on the results of our clinical observations and the liver function tests and of our studies on dietetic treatment and prognosis of liver cirrhosis cases.

Clinical Findings

Subjects

As in the previous report, 61 cases of liver cirrhosis hospitalized at our clinic during the period of 1920 to 1957 were the subjects of our study. Of these, 23 cases belonged to the pre-war and wartime cases studied between 1920–43 and 38 cases to the post-war cases treated between 1946–57. For studying the later results, the cause and the date of death after their discharge from our clinic, individual survey cards were prepared and filled in by answers from the subjects through a postal inquiry.

Findings and Discussion

I. Clinical signs and symptoms

The initial signs and symptoms are as shown in Table I. The time elapsed between the first occurence of the disease and the hospitalization averaged 5 months, 24 days in the pre-war cases and 4 months, 21 days in the post-war cases.

The clinical signs and symptoms are shown in Table II. The mean length of time elapsing between the occurrence of cirrhosis and the first appearance of ascites was 4 months, 21 days in the pre-war cases as well as the post-war cases, and ascites was detected within one year after the occurrence of liver cirrhosis in 90%. Edema was often observed con-

TABLE I

Initial Signs and Symptoms in 61 Cases of Liver Cirrhosis

(Per cent Incidence)

Signs and symptoms	Before 1945 (23 cases)	After 1945 (38 cases)	Total (61 cases)
Swollen abdomen	69.5	39.5	51.0
Edema of legs	30.5	10.3	18.1
Abdominal pain	13.1	18.4	16.4
Malaise	4.4	23.7	16.4
Enlarged liver	0	21.2	13.1
Ascites	4.4	10.6	8.4
Fever	4.4	7.9	6.6
Jaundice	0	5.3	3.3
Anorexia	0	5.3	3.3
Hematemesis	4.4	2.6	3.3

TABLE II

Clinical Signs and Symptoms in Liver Cirrhosis

(Per cent Incidence)

Signs and symptoms	Before 1945 (23 cases)	After 1945 (38 cases)	Total (61 cases)
Ascites	100.0	58.0	73.6
Palpable liver	47.8	84.5	70.5
Swollen abdomen	74.0	63.0	67.1
Edema	82.8	52.5	64.0
Fever	65.2	65.8	65.6
Anorexia	61.0	47.5	52.4
Jaundice	43.5	47.5	46.0
Dilated veins	47.8	42.2	44.2
Abdominal pain	43.5	39.5	41.0
Palpable spleen	21.7	34.2	29.5
Hepatic coma	30.5	23.7	26.2
Hematemeses	34.8	18.4	24.6
Bleeding tendencies	21.7	26.3	24.6
Esophageal varices	—	26.7	26.7
Abdominal distress	8.7	21.2	16.4
Nausea and vomiting	8.7	10.6	9.8
Vascular spiders	—	18.2	18.2
Impotence	4.4	5.3	4.9
Gynecomastia	—	7.9	7.9
Obesity	0	5.3	3.3

currently with ascites. Such edema appeared before, just at the same time, and after the occurrence of ascites in nearly the same frequency, as Wang has already reported.[2)]

II. Results of liver function tests

The rates of abnormalities found in liver function tests are as shown in Table III. Among the subjects with ascites, abnormal values in serum

TABLE III

Liver Function Tests

	Range of abnormal values	Without ascites		With ascites		Total	
		No. of cases examined	Per cent of cases showing abnormal value (%)	No. of cases examined	Per cent of cases showing abnormal value (%)	No. of cases examined	Per cent of cases showing abnormal value (%)
B.S.P.	>10%	16	75.0	17	94.1	33	85.0
Gros's test	>±	16	56.3	17	100.0	33	78.7
Serum albumin	<3.75 g/dl	12	33.3	15	100.0	27	70.5
C.C.F.	>+	15	53.3	14	85.7	29	69.1
T.F.T.	>+	15	53.3	14	78.6	29	65.6
Serum γ-globulin	>1.75 g/dl	12	25.0	15	86.6	27	63.0
T.T.T.	>4.0 units	15	40.0	14	78.6	29	58.6
Urine urobilinogen	>+	12	33.3	18	61.1	30	50.0
Icterus index	>10	16	31.3	17	64.8	33	48.5
Urine bilirubin	>±	6	16.7	14	28.6	20	25.0
Alkaline phosphatase	>4.0 units (Bodansky)	8	75.0	4	100.0	12	83.5
Mucoprotein	<2.43 g/dl	3	66.6	4	25.0	7	42.8

albumin, Gros's reaction, bromsulphalein test, serum γ-globulin, cephalin cholesterol flocculation, thymol flocculation and thymol turbidity were found in more than 79% of them, but among those without ascites only bromsulplalein test was found positive in 75% of the cases, abnormal results otherwise being found only in 25–56% of the cases.

Ascites was always present in the cases of less than 2.6 g/d*l* of serum albumin concentration and never in the cases with albumin concentration of 3.7 g/d*l* or above, the cases showing serum albumin level ranging between 2.6 and 3.7 g/d*l* either having or being free of ascites. The results of liver function tests revealed that among the 8 cases of cirrhosis caused by hepatitis, 6 showed abnormal values of thymol flocculation, but none of the 5 cases of the disease caused by overdrinking or drinking plus hepatitis showed such abnormal values, the rates of positivity of the two groups

showing stochastically significant difference. Some authors have reported on similar findings on thymol turbidity,[3)4)5)] but in our study, the number of tested cases was perhaps too small to reveal significant difference by causes of the disease in this respect. We could not confirm differences in other tests in the cases of liver cirrhosis attributed to different causes.

III. Complication with hepatoma

In Japan, liver cirrhosis is often complicated with hepatoma. In our 38 cases of cirrhosis examined histologically, hepatoma was diagnosed in the frequency of 18.4% (12.5% among the pre-war and 20% among the post-war cases)—a finding in fairly good agreement with the figures reported by Ueda *et al.*[6)]

IV. Treatment

In general, now we are chiefly relying on rest and high-protein, high-caloric diets plus administration of lipotropic factors, vitamins, glucuronic acid and glucose for the treatment of liver cirrhosis. In the cases with ascites or low serum albumin level, low-salt diet, diuretics, abdominal parecentesis and blood transfusion are used while in the cases with hepatic coma, low-protein diet, antibiotics including chlortetracycline and tetracycline, ACTH, cortisone, prednisolone and sodium glutaminate are given, and in the cases of hematomesis and melena, blood transfusion is resorted to. The improvements in the clinical findings, liver function test results and findings on liver biopsy noted after administration of such measures, may be summarized as follows:

1) Results at the time of discharge

In pre-war days, the mean duration of hospitalization was 29 days, but after the war, it rose to 79 days. Upon comparison of the findings at discharge from our clinic of the pre-war cases and those of the cases subjected to high-protein diet since 1951, we found that improvement in clinical findings and liver function test occurred in 26.7% of the latter, but none of the pre-war cases showed any improvement in their clinical findings. Death was the fate in 59% of the pre-war cases but in the cases treated in 1951 and later, the lethality was conspicuously reduced to 16.7%.

2) Changes of clinical findings during hospitalization

A comparison of the frequency of some clinical signs and symptoms observed at the times of entrance to and discharge from our clinic in patients treated with high-protein dieting since 1951, as shown in Fig. 1, reveals a stochastically significant difference in the frequency of fever, jaundice, tenderness of the liver and edema. Ascites and hemorrhage, in the cases showing such symptoms at the time of hospitalization, were significantly less frequent at the time of discharge, while we encountered no case that began to show any symptom of ascites or hemorrhage during

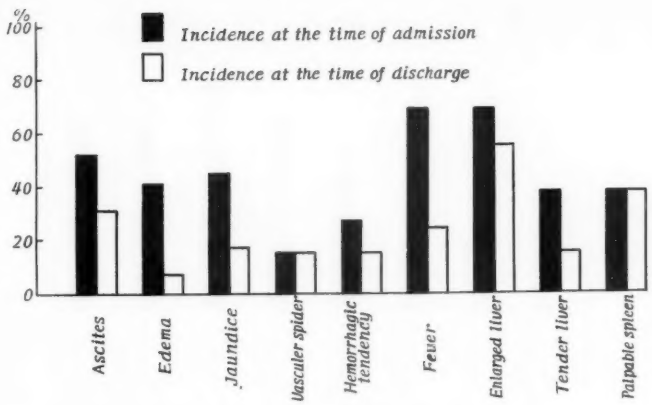

Fig. 1. Signs and Symptoms.

hospitalization.

From the above findings, it is clearly demonstrated that our treatment consisting in high-protein diet etc. has been effective in reducing the frequency of pyrexia, jaundice, tenderness of the liver, edema, ascites and tendency to hemorrhage, and thus improving the clinical symptoms during hospitalization. On the other hand, no change in the frequency of splenomegaly and vascular spiders was observed and the reduction of liver enlarge ment was not ascertainable during hospitalization.

Hepatic coma was seen in 4 cases during hospitalization. Antibiotics, ACTH or cortisone were applied to 3 of them, but therapeutic efficacy was observable in only one of the cases.

3) Changes in liver function tests

A comparison of the results of liver function tests at the time of admission to and discharge from our clinic revealed almost no improvement, unlike the case with clinical symptoms (Fig. 2). We may simply state that the Gros's reaction and bromsulphalein test were found slightly tending to normalize, thymol turbidity and thymol flocculation values were most difficult to improve.

4) Changes in liver biopsy findings effected by treatment

A comparison of the results of liver biopsy examination before and after treatment in 6 cases of liver cirrhosis revealed that in only one case of cirrhosis due to overdrinking of alcoholic beverages, improvement in histological findings, including reductions of fatty metamorphosis, of degeneration of liver cells and of fibrosis, was clearly observable but in some other cases, fibrosis was even found intensified after the treatment, so that in our general observation no tendency to diminution in fibrosis could be

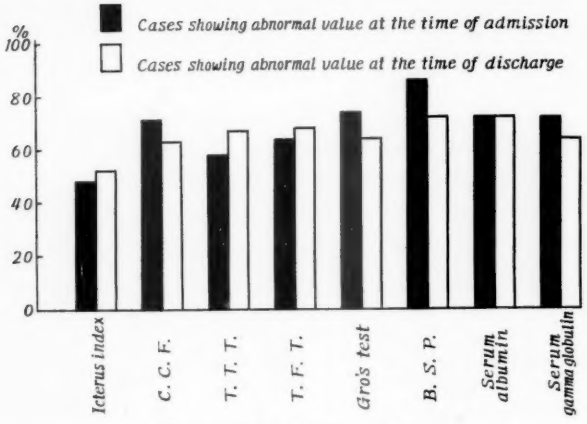

Fig. 2. Liver function tests.

confirmed and we failed in demonstrating general improvement in the histological findings.

Thus, in our patients, most of the general and the clinical findings were improved during hospitalization by high-protein dieting, but no perceptible improvement was effected in their liver function tests and pathohistological findings, so that we were led to infer that most of the pathological changes in these patients were advanced to the grade of irreversibility.

V. Prodnosis

For ascertaining how the prognosis of the liver cirrhosis patients was improved after high-protein high-calorie diet, the results obtained with pre-war cases and post-war cases were studied in comparison.

1) Rate of survival after the outbreak of liver cirrhosis

As shown in Fig. 3, of the cases who had ascites at the time of hospitalization only 32% survived one year and in 4 years all were dead, in pre-war days; but after the war, 62% survived one year and 27% were living even 4 years later. The difference in the 4 years' survival rates is stochastically significant, clearly indicating the improvement of prognosis in the post-war cases. The one year and 4 years' survival rates of the post-war cases free of ascites were 92% and 65% respectively, reflecting that the prognosis is more favorable in ascites-free cases.

2) Survival after occurrence of ascites

As shown in Fig. 4, the survival rates one year and 4 years after occurrence of ascites stood at 21% and 0% respectively in the pre-war cases, but in the post-war cases, they rose to 43% and 25% respectively, the difference in the 4 years' rates being stochastically significant, clearly reflecting the improvement of prognosis effected by high-protein dietetics.

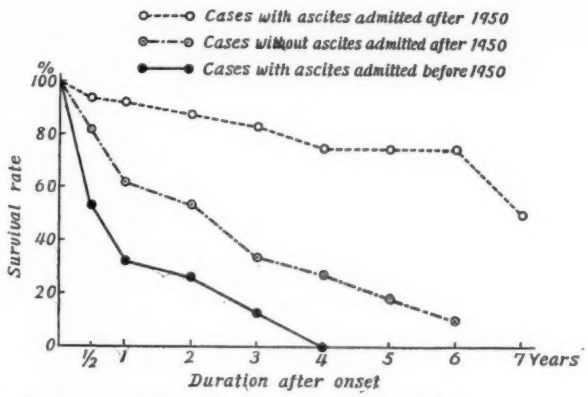

Fig. 3. Survival rate after the onset of liver cirrhosis.

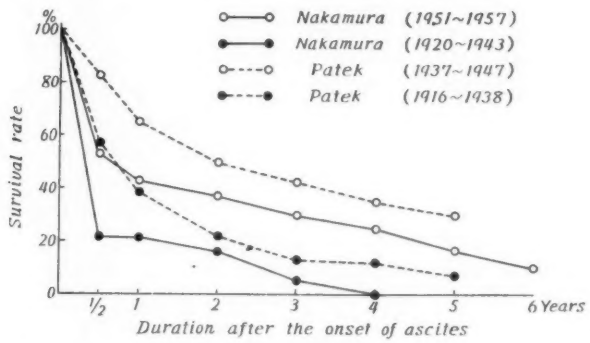

Fig. 4. Survival rate after the onset of ascites.

Comparing our survival rates after ascites with those of Patek *et al.*,[7] we find that the latter are somewhat higher, but in the finding of improved prognosis after the high-protein dietetics, our results are in total agreement with those obtained by Patek *et al.*

3) Clinical signs and symptoms and prognosis

The relation between the individual clinical signs and symptoms observed at the time of hospitalization and the prognosis in patients treated with high protein dieting since 1951 is as shown in Table IV. The survival rates after one year of the cases with and without enlarged liver were 94% and 14%, respectively, shwoing a stochastically significant difference, and reflecting the more favorable prognosis in the cases with enlarged liver. But no such significant correlation could be established between any other individual clinical sign or symptom and the prognosis. The

TABLE IV

Clinical Signs and Symptoms and Prognosis in Cirrhotic Patients Treated since 1951

Symptoms \ Duration after admission	One year			Two years		
	Alive	Dead	Survival rate (%)	Alive	Dead	Survival rate (%)
Hepatomegaly	15	1	94	10	2	83
Jaundice	8	3	73	7	3	70
Splenomegaly	7	3	70	5	3	63
Vascular spiders	2	1	67	1	1	(50)
Edema	5	4	56	3	5	38
Ascites	5	5	50	4	7	36
Gynecomastia	1	1	(50)	1	1	(50)
Esophageal varices	3	3	50			
Hematemeses	1	2	33	1	2	33
Total cases	16	7	70	10	8	56

prognosis in the cases with hematemesis, esophageal varices, ascites or edema appeared to be less favorable than in the cases free of such symptoms though the difference was not significant, while it was abvious that the correlations of jaundice, splenomegaly and vascular spider to the prognosis were not present.

4) Liver function tests and prognosis

Of the results of liver function tests at the time of hospitalization, the serum albumin concentration, the serum γ-globulin concentration and cephalin cholesterol flocculation were found significantly correlated with the prognosis, and in particular, the parallelism between the serum albumin concentration and the prognosis was most perceptible (Fig. 5). None of the other liver function tests showed any significant correlation to the survival rates 1 and 2 years after hospitalization.

The correlation between the results of liver function tests at the time of discharge and prognosis was found more frankly perceptible. In cases where low serum albumin concentration observed at the time of hospitalization was improved by treatment, the prognosis was also improved.

The results of liver function tests at the time of discharge from hospital and the survival rate one year after are shown in collation in Table V. The value showing the best parallelism with the prognosis is serum albumin concentration, Gros's reaction, cephalin cholesterol flocculation, icterus index, bromsulphalein test, thymol flocculation and serum γ-globulin content following it in this respect. Vide Fig. 6 (serum albumin), Fig. 7 (cephalin cholesterol flocculation) and Fig. 8 (bromsulphalein test).

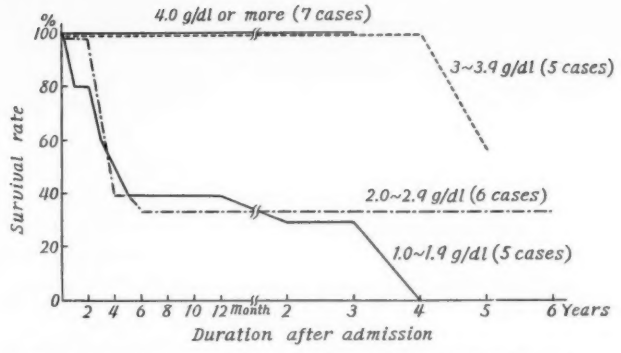

Fig. 5. Survival rate and serum albumin determined at the time of admission.

TABLE V

Liver Function Tests and Survival Rate 1 Year after Discharge in Cirrhotic Patients Treated since 1951

		No. of coses	Survival rate 1 year after discharge
Albumin	≧3.0 g/dl	9	100%*
	<3.0 g/dl	8	25%*
γ-Globulin	<2.0 g/dl	8	88%†
	≧2.0 g/dl	9	44%†
Gros's test	−~+	14	93%*
	++~+++	7	29%*
C.C.F.	−~+	8	100%*
	++~+++	9	33%*
T.T.T.	≦4.0 units	8	88%
	>4.0 units	9	44%
T.F.T.	−~+	6	100%*
	++~+++	11	45%*
Icterus index	≦10	8	100%*
	>10	12	50%*
B.S.P.	≦30%	13	92%*
	>30%	7	29%*

* The difference is stochastically significant.

† Although the difference of survival rate after 1 year is not stochastically significant, that after 3 or 4 months is stochastically significant.

No significant correlation was found between thymol turbidity and the prognosis.

Thus, the values obtained by liver function tests are found very closely correlated with the prognosis of liver cirrhosis cases, so that we can say such liver function tests are not only of service in diagnosis of liver cirrhosis

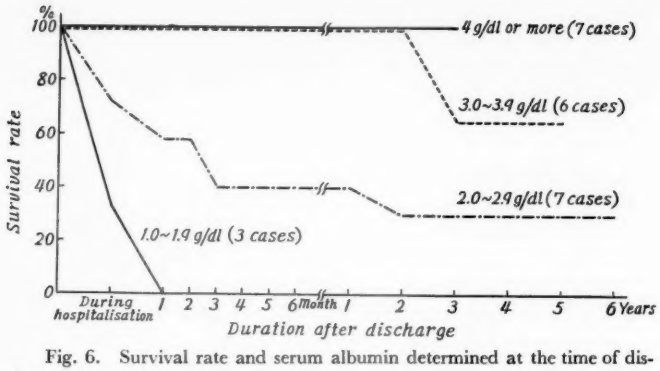

Fig. 6. Survival rate and serum albumin determined at the time of discharge.

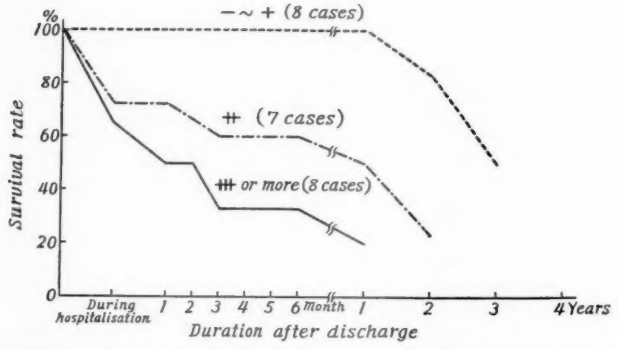

Fig. 7. Survival rate and cephalin cholesterol flocculation determined at the time of discharge.

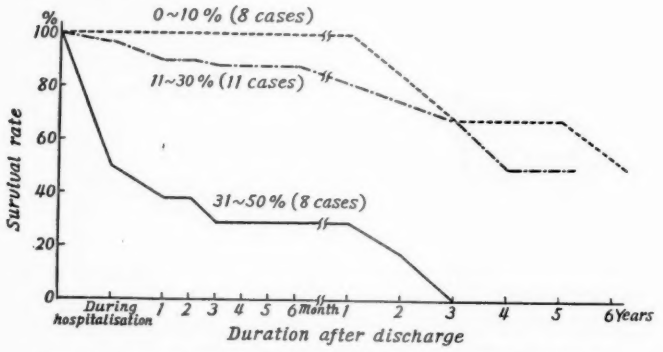

Fig. 8. Survival rate and bromsulphalein test determined at the time of discharge.

but also in estimating the prognosis of the patients, as Moser *et al.*[8] have already pointed out.

5) Causes of the disease and prognosis

The prognosis of the cases of cirrhosis due to overdrinking seemed to be more favorable than that of the cases due to hepatitis, but the number of the cases was too small to bring out a stochastically significant difference.

6) Prognosis after complicating hepatoma

The above described post-treatment and later results of our liver cirrhosis cases are given in exclusion of the cases with hepatoma in complication. The prognosis of such cases with hepatoma was very unfavorable, all the cases dying within 4 months after hospitalization without exception.

7) Causes of death

The direct causes of death of liver cirrhosis cases were ranged in the descending order of frequency of hepatic coma, hematemesis, and miscellaneous (Table VI). The rate of death due to hematemesis and hematochezia among the post-war cases, if cases with complicating hepatoma are included, is 17.6% and smaller than that among the pre-war cases, but this is due to the fact that the post-war cases include the fatal cases with hepatoma to the ratio of 1/3 of the total; so that if these are counted out, the rate rises to 27.2% and is not much different from that in pre-war cases. Upon studying the relation between the causes of cirrhosis and of death, no significant correlation could be established.

TABLE VI

Causes of Death in Liver Cirrhosis

Figures in parentheses are percentages.

Causes of death	Including cases with primary liver carcinoma		Excluding cases with primary liver carcinoma	
	Before 1945	After 1945	Before 1945	After 1945
Hepatic coma	7 (35.0)	9 (53.0)	7 (36.8)	6 (54.6)
Hemorrhage	7 (35.0)	3 (17.6)	7 (36.8)	3 (27.2)
General prostration	3 (15.0)	2 (11.8)	3 (15.8)	0
Infection	1 (5.0)	0	1 (5.3)	0
Post-operative	0	3 (17.6)	0	2 (18.2)
Unknown	2 (5.0)	0	1 (5.3)	0

SUMMARY AND CONCLUSIONS

1. Of the patients having liver cirrhosis with ascites, more than 79% showed abnormal values in the serum albumin concentration, Gros's

reaction, bromsulphalein test, serum γ-globulin concentration, cephalin cholesterol flocculation, thymol flocculation and thymol turbidity, but of the cases free of ascites 75% showed positive value of bromsulphalein test, abnormalities in the results of the other liver function tests being observed in 25–56% of the cases.

Abnormality of thymol flocculation was more frequent in the cases of cirrhosis due to hepatitis than in the cases due to alcoholic intake, showing significant difference. The other liver function tests did not show such clear difference by causes of the disease.

2. Complication of cirrhosis with hepatoma was observed in the frequency of 18.4% of the 38 cases subjected to biopsy or autopsy examinations (12.5% among the pre-war and 20% among the post-war cases).

3. Improvement in clinical symptoms effected by high-protein dietetics was obvious, but little improvement was seen in the results of liver function tests or the liver biopsy findings.

4. The survival rates 4 years after the outbreak of liver cirrhosis stood at 27% and 65% in the post-war cases with or without ascites respectively, indicating a very marked improvement in prognosis, as compared with the rate of 0% in pre-war patients. Of the post-war cases without ascites 65% survived 4 years later.

5. The prognosis agreed better with the results of liver function tests, especially those at the time of discharge, than with the clinical findings during hospitalization, all values other than thymol turbidity being found in parallism with the prognosis.

References

1) Nakamura, T., Nakamura, S., Kimura, Tohoku J. Exp. Med., To be published.
2) Wang, Chinese J. Med., 1936, **50**, 891.
3) Baggenstoss & Stauffer, Gastroenterology, 1952, **22**, 157.
4) Watson, *ibid.*, 1947, **9**, 752.
5) Mann, Snell & Bitt, *ibid.*, 1947, **9**, 651.
6) Ueda & Horiguchi, Saishinigaku (Jap.), 1958, **13**, 668.
7) Patek, Post, Ratnoff, Mankin & Hillman, J. Amer. Med. Assoc., 1948, **138**, 543.
8) Moser, Rosenak, Pickett & McIntire, Gastroenterology, 1951, **18**, 86.

The Tohoku Journal of Experimental Medicine, Vol. 69, Nos. 2-3, 1959

Chemical Nature of Toxohormone (Nakahara)

Eighth Report. Toxohormone AP II_1 and II′ from Cancerous and Noncancerous Ascitic Fluids*

By

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(Received for publication, January 15, 1959)

Formerly we[1] mentioned that two toxohormones, one KIK-active and the other KIK-inactive, are secreted in urine of not only cancer patients but also normals. And, from gastric cancer as well as non-cancered gastric mucosa were isolated two different toxohormones[2], although both are group active mucopolysaccharides. Thus it might be said that generally one secretion or one tissue contains more than one kind of toxohormone. Regarding the cancerous ascitic fluid, we have detected so far three toxohormones in it, one of which is AP I[3] a globulin possessing simultaneously KIK potency, a second the KIK-active glucidamin reported by Sugimoto[4] and a third a globulin closely resembling AP I but KIK-inactive.

The present account deals with the last toxohormone from cancerous ascitic fluids with original seat in stomach (AP II_1) as well as a corresponding protein from ascitic fluids due to liver-cirrhosis as a representative kind of noncancerous ascitic fluid (AP II′).

Experimental

Preparation Procedure of the Toxohormones

For each of the two kinds of toxohormone, one example of preparation runs will be described.

AP II_1, a toxohormone-active globulin other than AP I from cancerous ascitic fluids with original seat in stomach

First stage. 4.3 *l.* of combined cancerous ascitic fluids (light yellow, transparent ; pH 8.2 ; d 1.015) from a stomach cancer patient was distilled in vacuo to about 800 cc., and the condensate was dialyzed against running water for 3 days, transferred to the middle compartment of a Pauli apparatus without antecedently separating off some precipitate, that had occurred

* 14th report of Masamune and co-workers' " On Proteins and Amino Acids ".

during dialysis, and electrodialyzed until no more flow of electricity. The precipitate increased enormously thereby. The whole deposit was centrifuged, washed with ethanol and ether in succession and dried in vacuo over anhydrous calcium chloride. 42 g. of a gray powder (ascitic globulins =AG) was obtained.

Second stage. 42 g. of AG was pulverized, suspended in 600 cc. of distilled water, and after addition of 10% NaOH to pH 9.0, stood in a refrigerator (0–3°C) additionally adding the NaOH solution from time to time to maintain the pH value at 8.5–9.0. After 5 days, the mixture was supplemented with water to 800 cc. and centrifuged in a refrigerated centrifuge (0°C), and the opalescent supernatant (165 cc.) was neutralized with 1 *N* HCl and freed from some precipitate that had occurred thereby. 29.7 g. (18 g. per 100 cc. of the solution) of sodium chloride was added to the centrifuged supernatant (163 cc.), and this mixture was placed in the refrigerator for one day with occasional agitation and acidified to pH 4.2 with 1 *N* HCl. The abundant white precipitate was centrifuged off at 0°C and washed with two 60 cc. portions of 18% NaCl of pH 4.2. It was next suspended in 200 cc. of distilled water, adjusted to pH 8.5 with 5% NaOH and freed from sodium chloride by 3 days' dialysis against running water. The solution which had changed intensively cloudy was again alkalinized to pH 9.0, and the insoluble part was discarded after centrifugation. The supernatant here was neutralized with 2 *N* HCl exactly to pH 7.0, the small precipitate was eliminated, and after making up to 165 cc., the solution was subjected to alcoholic fractionation (next stage).

Third stage. 55 cc. of the neutral solution above was diluted to 100 cc. and chilled on ice, and abs. ethanol was added under vigorous agitation in such portions as each increased the ethanol concentration by 10% (v/v), centrifuging off every deposit in the refrigerated centrifuge after 2–3 hours. The centrifugates were separately washed with ethanol and ether and dried as above, with the yields of fractions:

Fraction No.	I	II	III	IV	V	VI	VII
Ethanol concn. (% (v/v))	0–10	10–20	20–30	30–40	40–50	50–60	60–70
Yield (mg.)	30	13	10	45	135	680	120

The balance of the solution at the end of the second stage was also treated in a similar manner, and the fractions which precipitated at 40–50% ethanol and those at 50–60% ethanol were united together. The total amount given was 2.4 g.

Fourth stage. As AG was in the second stage, 2.4 g. of the product was taken up in 200 cc. of water by means of alkali, and the part that was insoluble at pH 9.0 and that precipitated on shifting the hydrogen ion

concentration to pH 7.0 were eliminated. The light opalescent centrifuged supernatant (neutral) was made up to 240 cc. with water and fractionated by addition of 6.6 g. portions of solid ammonium sulfate, each of which increased the concentration of this salt by 0.2 *M*, in the manner alcoholic fractionation was carried out above. The fractions precipitated here were separately dissolved in 70–150 cc. of water and dialyzed against running water for 3–4 days to remove ammonium sulfate, employing toluene as a preservative. Then, the solutions were distilled under reduced pressure to syrupy consistency, dehydrated with excessive ethanol, washed with ethanol and ether and dried as above with yields:

Fraction No.	I, II, III	IV	V	VI	VII	VIII	IX	
Molarity of ammonium sulfate (*M*)	0–0.4, 0.4–0.6, 0.6–0.8	0.8–1.0	1.0–1.2	1.2–1.4	1.4–1.6	1.6–1.8	1.8–2.0	2.0...3.0
Yield (mg.)	0	64	1080	373	297	85	75	157

Frs. V–VII were dissolved together in 130 cc. of water in the same manner as AG was in the second stage (Here almost no precipitate occurred when the solution of pH 9.0 was neutralized), and the neutral solution was made up to 160 cc. and fractioned by successive addition of 4.22 g. portions of ammonium sulfate as above. The deposits were freed from the salt, condensed, precipitated, washed and dried also as above. The yields here amounted as follows:

Fraction No.	I	II	III	IV	V
Molarity of ammonium sulfate (*M*)	0.6–0.8	0.8–1.0	1.0–1.2	1.2–1.4	1.4–1.6
Yield (mg.)	37	141	755	424	117

Fifth stage. 1.1 g. of united Fr. III and IV was suspended in 100 cc. of water, dissolved by the aid of NaOH also as AG was in the second stage and electrodialyzed, and the deposit thereby was centrifuged off, washed with water (about 60 cc.), and after again dissolving in 40 cc. of water by addition of 1 *N* NaOH to pH 7.4, freeze-dried. A white, light powder weighing 0.95 g. was given—**AP II$_1$**.

AP II′, a corresponding protein fraction of ascitic fluids from liver cirrhosis patients as a representative kind of non-cancerous ascitic fluid

Starting with 1.6 *l.* of an ascitic fluid (yellowish, transparent ; pH 8.2 ; d 1.015) from a liver-cirrhosis patient, 0.62 g. of a white, light powder corresponding to AP II$_1$—**AP II′**—was obtained in a manner similar to above without setting aside the intermediate products partially. The main part of the toxohormone here fell on the corresponding fractions to those above in fractionation with ethanol as well as in fractionations with ammonium sulfate.

Properties of the Preparatoins

1) Homogeneity. AP II_1, and II′ gave each a single, symmetrical boundary after electrophoresis in either of an alkaline (pH 8.6) and an acid (pH 2.0) buffer. Their mobilities in the alkaline buffer were not the same. The examination was made in a Tiselius apparatus of HTD-1 type Hitachi. See Fig. 1.

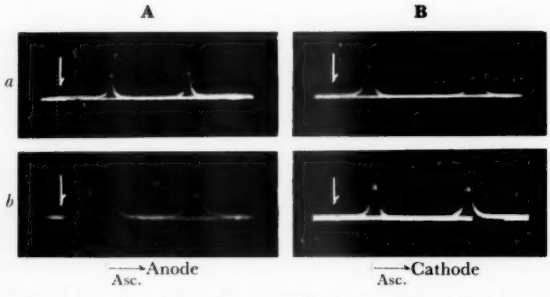

Fig. 1. Electrophoresis patterns (×2/3) of 1% solutions in a veronal-HCl buffer of pH 8.6 and I 0.2 (**A**) and a glycine-HCl buffer of pH 2.0 and I 0.2 (**B**) of AP II_1 (*a*) and AP II′ (*b*) ; current 5 mA ; temperature 10°C ; exposure 120 minutes after starting current. Mobility :

AP II_1 pH 8.6 $\dfrac{(-1.15)\times 0.01043\times 0.18}{120\times 60\times 0.005} = -6.00\times 10^{-5}$ cm²/volt/sec

pH 2.0 $\dfrac{(-0.80)\times 0.01544\times 0.18}{120\times 60\times 0.005} = -6.18\times 10^{-5}$ "

AP II′ pH 8.6 $\dfrac{(-1.08)\times 0.01043\times 0.18}{120\times 60\times 0.005} = -5.63\times 10^{-5}$ "

pH 2.0 $\dfrac{(-0.92)\times 0.01544\times 0.18}{120\times 60\times 0.005} = -7.10\times 10^{-5}$ "

The homogeneity of the preparations was further ascertained by column chromatography :— A 1.1×20 cm. column of a strong anion exchanger Dowex 1×2 (200–400 mesh) was equilibrated with 0.025 *M* borax-HCl buffer of pH 8.5, and 2 or 1 cc. of the same buffer containing 20 mg. of AP II_1 or 10 mg. of AP II′ respectively was added, and washed with also the same buffer at a flow rate of 40 drops per hour. The eluate was collected in fractions of 20 drops (about 2 cc.) and analyzed by a biuret reaction (Gornall *et al.*[5]) and measurement of ultraviolet absorption at 280 mμ. The results are diagrammed in Fig. 2, showing a single symmetrical peak of concentration in a definite range of fractions for either of the substances. Namely, the preparations again proved homogeneous.

2) Toxohormone activity. It was assayed by our usual method[3]. As shown in Table I, the minimum toxohormone active dosis of AP II_1 and II′ were found about 2 and 4 mg. respectively.

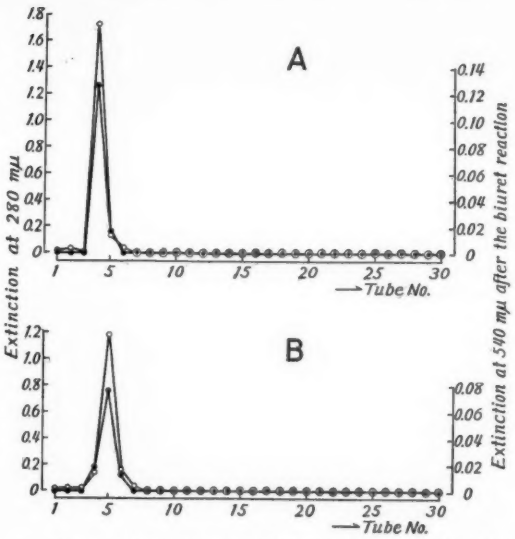

Fig. 2. Column chromatograms of 20 mg. of AP II_1 (**A**) and 10 mg. of AP II′ (**B**) developed by the aid of a Dowex 1×2 column. -○—○- Extinctions at 280 mμ. -●—●- Extinctions at 540 mμ after the biuret reaction.

Table I-A

Liver-catalase Depression *in vivo*

The average O_2 volume evolved for each dosis in each test-set is expressed in per cent of the corresponding O_2 volume in the blank control.

Assay No.	Number of mice used	Amount of substance injected (mg.)			
		5	3	1	0.5
By AP II_1:					
1	12		62.8	76.0	91.4
2	9		58.8		70.9
3	9		65.3	80.3	
4	9			76.6	82.2
5	6		72.7		
6	6			79.1	
7	9			83.5	91.1
Average			64.9	79.1	83.9
By AP II′:					
8	9	74.4		75.2	
9	12	63.4	77.9	85.3	
10	12	64.8	70.3	86.1	
11	9	68·5	82.7		
12	9	71.4	73.2		
Average		68.5	76.0	82.2	

TABLE I-B

Effect *in vivo* on Kidney- and Blood-catalase

Assay No.	Kidney	Blood	Liver
Effect of AP II_1 :			
	Oxygen evolved (cc.) in blank experiment		
201	5.2	1.6	15.8
202	5.3	1.9	15.5
203	3.5	2.2	12.3
Average	4.3	1.9	14.5
	Oxygen evolved (cc.) in main experiment		
204	4.0	1.9	10.9
205	4.9	1.7	10.6
206	5.1	2.0	10.2
Average (% of control value)	4.7 (109)	1.9 (100)	10.6 (73)
Effect of AP II′ :			
	Oxygen evolved (cc.) in blank experiment		
207	5.1	2.4	13.5
208	5.8	2.0	13.3
209	4.4	2.4	14.6
Average	5.1	2.3	13.8
	Oxygen evolved (cc.) in main experiment		
210	4.5	2.3	11.2
211	5.3	2.2	7.9
212	5.7	2.5	10.7
Average (% of control value)	5.2 (102)	2.3 (100)	9.9 (72)

3) KIK activity.[4] The method Masamune and Kawasaki described[6] was applied. The result is illustrated in Table II. The substances are regarded as KIK-inactive, because neither of them induced anemia in rabbits even when injected intravenously in dosage of 1.5 mg. per kg. body weight.

4) Paper chromatography of the sugars and amino acids constituting the preparations. a) Sugars. 10.6 mg. of AP II_1 was heated with 1 cc. of 1 *N* sulfuric acid in a sealed tube at 100°C for 5 hours. About 3.5 cc. of the satd. baryta was added to the hydrolysate until a pH value of 4.0, and the

TABLE I-C

Effect *in vitro* on Liver-catalase

Assay No.	Liver-catalase activity (oxygen evolved in cc.)		
	Control I (No incubation)	Control II (Incubated without toxohormone)	Main (Incubated with toxohormone)
Effect of AP II_1 (3 mg.) :			
301	13.8	11.8	12.0
302	12.5	9.0	8.9
303	16.5	10.5	10.3
Average	14.3	10.4	10.3 (99% of Control II value)
Effect of AP II′ (5 mg.) :			
304	16.5	11.3	10.8
305	15.2	11.5	11.8
306	12.0	8.4	9.2
Average	14.6	10.4	10.6 (102% of Control II value)

TABLE II

Anemia-inducing Potency of AP II_1 and II′

Assay No.	Rabbit			Amount of substance injected* per kg. body weight (mg.)	Number of red cells ($\times 10^4$, per mm^3)						
	No.	Body wt. (kg.)	Sex		Before inj.	Time after inj. (hrs.) 1	2	3	4	5	Maximum decrease (%)
The potency of AP II_1 :											
1	35	2.0	♀	1.5	551	568	559	601	540	*534*	4
2	36	2.2	♂	1.5	533	521	544	*479*	496	492	8
The potency of AP II′ :											
3	35	2.0	♀	1.5	590	588	579	589	587	*569*	4
4	36	2.2	♂	1.5	750	790	*739*	757	777	755	1

* Intravenously.

precipitate was centrifuged off and discarded after washing with warm water. The supernatant and washing were evaporated to about 1 cc. by standing in vacuo over anhydrous calcium chloride, and the deposit occurring thereby was filtered off and washed. The filtrate and washing here were distilled to dryness, and the still-residue was dissolved in 0.1 cc. of

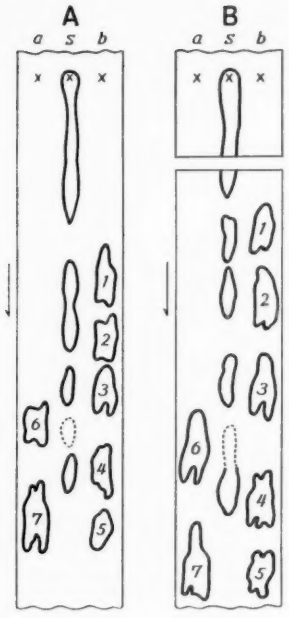

Fig. 3. Sugar paper chromatograms (×2/5) of the hydrolysates of AP II_1 (**A**) and of AP II′ (**B**) developed with solvent butanol-pyridine-water (5:3:2); slivers 60×7 cm., Toyo Roshi filter paper; irrigation descending; temperature of test: AP II_1 25°±2°C, AP II′ 22°±1°C; duration of test: AP II_1 20 hrs., AP II′ 24 hrs. Reference runs effected with 1% solutions with respect to individual sugars. Indicator aniline hydrogen phthalate reagent of Partridge.

a, *b*: reference. *s*: main run. *1* galactosamine, *2* glucosamine, *3* galactose, *4* mannose, *5* L-fucose, *6* glucose, *7* xylose.

water and paper-chromatographed according to Masamune and Yosizawa[7]. As regards AP II′, 18.2 mg. of it was hydrolyzed, and the still-residue of the hydrolysate free from H_2SO_4 was dissolved in 0.1 cc. and chromatographed taking 0.04 cc. of the solution. See Fig. 3.

In either of the chromatograms, spots were shown at the positions corresponding to galactosamine, glucosamine, galactose and mannose. It is doubtful that the very faint spots at the positions corresponding to glucose are plausibly ascribed to this sugar.

b) Amino acids. 5.6 mg. of AP II_1 was sealed with 1 cc. of 6 *N* HCl in an ampoule and heated in a boiling water-bath for 24 hours. The hydrolysate was freed from the hydrochloric acid by repeated vacuum evaporations over solid caustic soda to dryness, adding water to every residue. The final evaporated residue was taken up in 0.18 cc. of water (The solution was not perfectly clear), and 0.03 cc. of the solution was chromatographed on a 30×30 cm. square of Toyo Roshi filter paper No. 50. Of AP II′, 6 mg. was hydrolyzed, and the still-residue of the hydrolysate freed from the free hydrochloric acid was dissolved in 0.24 cc. of water and chromatographed taking 0.04 cc. of the solution. Aspartic and glutamic acids, serine, glycine, threonine, alanine, tyrosine, phenylalanine, leucine and/or isoleucine, valine and/or methionine, proline, hexone bases and a

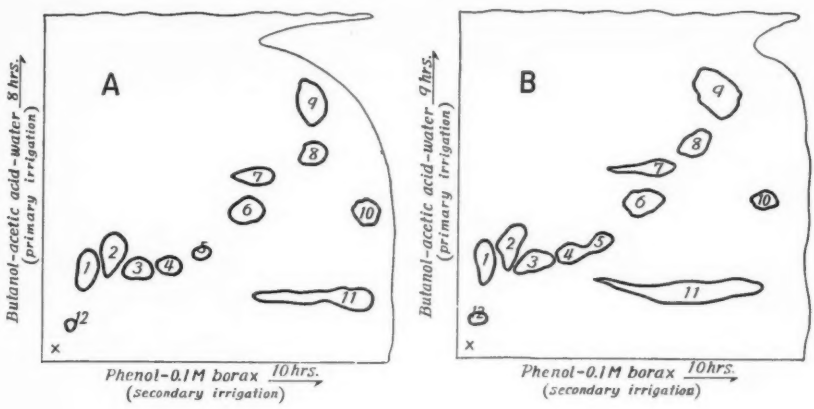

Fig. 4. Two-dimensional amino acid-paper chromatograms of the hydrolysates of AP II_1 (**A**) and of AP II′ (**B**), developed by employing solvent butanol-acetic acid-water (4:1:2) for the primary irrigation and solvent phenol-0.1 *M* borax (10:1) for the secondary irrigation. Paper square 30×30 cm., Toyo Roshi filter paper No. 50. Temperature of test 20–25°C. Spray reagent 0.2% ninhydrin in moist butanol.

1 aspartic acid, 2 glutamic acid, 3 serine, 4 glycine, 5 threonine, 6 alanine, 7 tyrosine, 8 valine and/or methionine, 9 phenylalanine, leucine and/or isoleucine, 10 proline, 11 hexone bases, 12 a probable peptide.

probable peptide were detected in AP II_1 as well as in AP II′.

5) *Test-tube tests.* Both of the preparations were strongly biuret positive and weakly Molisch positive.

6) *Quantitative analyses.* The analytical figures are embodied in Table III. No quantitative discrepancies are discerned between AP II_1 and II′ regarding hexosamine and hexoses, because these components are too small in content to give reliably accurate values. With respect to the amino acids, serine is present less and the total sum of phenylalanine, leucine and isoleucine as expressed as leucine more in AP II_1 than in AP II′.

7) *Ultraviolet absorption.* 0.5 mg% solutions in water were examined in a photoelectric spectrophotometer of EPU-2A type Hitachi. As shown in Fig. 5, ultraviolet absorption with maximum at 278 mμ occurred in similar strengths by the two proteins.

8) *Specific refraction.* 1% solutions (pH 8.0) of the preparations were examined in an Abbe refractometer at 17.5°C, giving resembling $[n]_D^{17.5}$ values :—

TABLE III

Composition in per cent of AP II_1 and AP II′

Analysis	AP II_1	AP II′
N*	14.4	14.4
Ash†	0.8	1.6
Hexose‡ (as galactose)		
PHR_2	2.1	1.4
$SMHR_2$	0.8	0.4
HR_{10}	2.5	1.8
Hexosamine§	2.3	1.6
Pentose//	0	0
Methylpentose¶	0	0
Sialic acid**		
ER	0	0
DR	0	0
Amino acids††:		
Aspartic acid	14.8	16.2
Glutamic acid	14.8	15.4
Serine	11.6	14.3
Glycine	6.4	7.5
Threonine	5.8	6.4
Alanine	6.2	5.8
Tyrosine	3.2	2.9
Valine and methionine (as valine)	7.0	6.4
Phenylalanine, leucine and isoleucine (as leucine)	16.0	12.2
Proline	6.6	6.0
Hexone bases (as lysine)	7.5	6.8

* Micro-Kjeldahl method. † Pregl method without use of H_2SO_4. ‡ Masamune and Sakamoto[8]. § Masamune and Yosizawa[9]. // Masamune and Sakamoto[8]. ¶ Masamune and Kawasaki[10]. ** Werner and Odin (direct Ehrlich method and diphenylamine method)[11]. †† A modification of Masamune and Hakomori method[12] (Solvent phenol–0.1 *M* borax (10:1) was replaced for solvent phenol–0.1 *M* borax (86:14)).

AP II_1:

$$n=1.33513,\quad n_0=1.33324,\quad d^{17.5}/_4=1.0035,\quad d_0{}^{17.5}/_4=1.0003$$

$$[n]_D^{17.5}=\frac{1.33513^2-1}{1.33513^2+2}\times\frac{1}{1.0035}\times\frac{100}{1.000}-\frac{1.33324^2-1}{1.33324^2+2}\times\frac{1}{1.0003}\times\frac{99}{1.000}=0.245$$

AP II′:

$$n=1.33547,\quad n_0=1.33324,\quad d^{17.5}/_4=1.0035,\quad d_0{}^{17.5}/_4=1.0003$$

$$[n]_D^{17.5}=\frac{1.33547^2-1}{1.33547^2+2}\times\frac{1}{1.0035}\times\frac{100}{1.000}-\frac{1.33324^2-1}{1.33324^2+2}\times\frac{1}{1.0003}\times\frac{99}{1.000}=0.264$$

In short, the cancerous toxohormone here and the corresponding

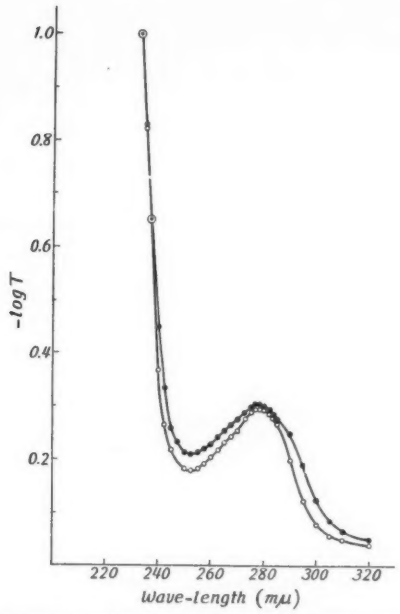

Fig. 5. Ultraviolet absorption spectra of 0.5 mg% aqueous solutions (pH 6.6) of AP II_1 (-○—○-) and of AP II′ (-●—●-).

noncancerous differ in composition in serine content, phenylalanine, leucine and isoleucine content as a whole and possibly also in electrophoretic mobility in so far as was examined chemical-analytically and physically.

Summary

1. There have been isolated toxohormones AP II_1 and II′ which are globulins in the cancerous ascitic fluid of stomach cancer patients and noncancerous ascitic fluid of liver cirrhosis patients respectively and both precipitate at 40 to 60% ethanol and at 1.0–1.4 *M* ammonium sulfate.

2. They were examined biologically, chemically and physically.

3. They are devoid of anemia-inducing potency.

4. The former protein is more strongly toxohormone active than the latter, while they differ from each other only regarding serine content and phenylalanine, leucine and isoleucine content as a whole and possibly also regarding electrophoretic mobility, in so far as was examined.

The Ministry of Education gave a grant in aid to us through the Grant Committee for Medical, Dental and Pharmaceutical Researches, which is gratefully acknowledged. H. Masamune.

References

1) Masamune, Kaketa & Abe, S., Tohoku J. Exp. Med., 1958, **67**, 329 ; Masamune, Hakomori, Kaketa & Abe, S., *ibid.*, 1958, **67**, 334 ; Masamune, Hakomori, Kaketa, Sinohara & Abe, S., *ibid.*, 1958, **68**, 63.

2) Masamune, Kawasaki, Abe, S., Oyama, & Yamaguchi, *ibid.*, 1958, **68**, 81 ; Kawasaki, *ibid.*, 1958, **68**, 119 ; 1959, **69**, 153.

3) Masamune, Tsuiki, Kamiyama, Abe, S., Haga, Kawasaki, Kaketa & Abe, Sh., *ibid.*, 1958, **67**, 309.

4) Sugimoto, *ibid.*, 1956, **64**, 271 ; Masamune, Hakomori, Sugimoto & Genda, unpublished.

5) Gornall, Bardawill & David, J. Biol. Chem., 1948, **177**, 751.

6) Masamune & Kawasaki, Tohoku J. Exp. Med., 1956, **63**, 369 (f. 372).

7) Masamune & Yosizawa, *ibid.*, 1953, **59**, 1.

8) Masamune & Sakamoto, *ibid.*, 1956, **63**, 345.

9) Masamune & Yosizawa, *ibid.*, 1957, **65**, 169.

10) Masamune & Kawasaki, *ibid.*, 1956, **63**, 369 (f. 381).

11) Werner & Odin, Acta soc. med. Upsal., 1952, **57**, 270.

12) Masamune & Hakomori, Tohoku J. Exp. Med., 1956, **64**, 59.

The Tohoku Journal of Experimental Medicine, Vol. 69, Nos. 2-3, 1959

Chemical Nature of Toxohormone (Nakahara)

Ninth Report. Toxohormones AP II_2, II_3, III_3 and IV_3 from Cancerous Ascitic Fluids*

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(Received for publication, January 15, 1959)

It was already mentioned that there are various toxohormones in one tissue or in one secretion[1)-5)] and at that the corresponding cancerous toxohormones from the same source of patients are identical irrespective of the site of original tumor[2)5)]. Most recently we have isolated AP II_2 and II_3 the protein fraction corresponding to AP II_1[6)] of ascitic fluids from a hepatoma patient and an ovarian cancer patient, and AP III_3 and IV_3 toxohormones from the same cancerous ascitic fluid with original seat in ovary which, however, were protein fractions different from AP II_3, to compare them with one another but also with AP II_1.

Experimental

Preparation Procedure

1) AP II_2

The procedure of preparing AP II_1 in the foregoing report[6)] was adapted to 3.5 *l.* of ascitic fluids combined (yellowish brown, clear; pH 7.0; d 1.020) from a hepatoma patient. Ethanolic fractionation was effected by raising ethanol concentration by 20% each time, and the precipitate at 40–60% (3.0 g.) was taken up in a volume a little less than 150 cc. of water by the aid of NaOH, followed by neutralization. The neutral opalescent solution was made up exactly to 150 cc. with water and fractionated with ammonium sulfate at first into 3 fractions, by adding stepwise of portions of this salt each corresponding to 0.8 *M* in the solution:

Fraction No.	I	II	III
Molarity of $(NH_4)_2SO_4$ (*M*)	0–0.8	0.8–1.6	1.6-satn.
Yield (g.)	0.73		0.13

* 15th report of Masamune and co-workers' "On Proteins and Amino Acids".

Afterwards, the precipitate at the range 0.8–1.6 *M* of the salt was refractionated without previous drying by addition of portions of the salt each increasing the concentration of the salt by 0.2 *M*.

Fraction No.	I′	II′	III′	IV′
Molarity of $(NH_4)_2SO_4$ (*M*)	0–0.8	0.8–1.0	1.0–1.2	1.2–1.4
Yield (mg.)	0	91	303	183

Fr. III′ and IV′ were united and freeze-dried as sodium salt—**AP II**$_2$.

2) AP II$_3$, *III*$_3$ *and IV*$_3$

2.4 *l.* of ascitic fluids combined (yellowish brown, transparent; pH 7.8; d 1.025) from an ovarian cancer patient was subjected at first to the first and second stage fractionations of ascitic fluid when AP II_1 was prepared.[6] The precipitates at the range 40–60% of ethanol, united and dried, were next fractionally precipitated here also with ammonium sulfate but with some modification:—

10.8 g. of the alcoholic precipitates were dissolved in 450 cc. of water by the aid of sodium hydroxide, added until pH 9.0, and after removing the part that had precipitated when the solution was readjusted to pH 7.0, made up to 500 cc., and ammonium sulfate was added in 52.8 g. portions as to increase the salt concentration by 0.8 *M* each time as in *1*), centrifuging off every precipitate on occurrence. The yields after dialysis of the precipitates were as follows.

Fraction No.	I	II	III	IV	V	
Molarity of $(NH_4)_2SO_4$ (*M*)	0–0.8	0.8–1.6	1.6–2.4	2.4–3.2	3.2–4.0	4.0-satn.
Yield (g.)	0.0	1.5	2.8	3.1	1.1	0.15

The precipitate at 0.8–1.6 *M* of ammonium sulfate (Fr. II) thus did not correspond in amount to the peak of the protein fractions, Fr. III and IV amounting more, contrary to usual cases of the ascitic fluids from gastric cancer- and hepatoma-patient. In the present case, therefore, Fr. III and IV were employed as extra materials to isolate homogeneous proteins with the aim of examining if new toxohormones are discovered.

i) 1.25 g. of Fr., II was suspended in 100 cc. of water, dissolved by addition of sodium hydroxide as above, neutralized to pH 7.0 and made up to 125 cc. with water. 3.3 g. portions of ammonium sulfate, increasing each the salt concentration of the solution by 0.2 *M*, were added in the manner above, and the precipitates at 1.0–1.4 *M* of the salt were freed from the salt and freeze-dried as sodium salt. 130 mg. of a fluffy powder was yielded—**AP II**$_3$.

ii) Fr. III (2.8 g.) was taken up in 280 cc. of water in a manner similar to above and fractionally precipitated with ammonium sulfate as in *i*). The precipitates at 1.6–2.0 *M* of the salt were freed from the salt,

changed into sodium salt and freeze-dried—**AP III$_3$**. Yield 125 mg.

iii) 3 g. of Fr. IV was dissolved in 300 cc. of water, ammonium sulfate was added in portions as above, and the fractions precipitated at 2.4–2.8 *M* of the salt were collected, freed from the salt by successive dialysis and electrodialysis, and freeze-dried as sodium salt—**AP IV$_3$**. Yield 370 mg.

Properties of the Preparations

Homogeneity. They were examined in a Tiselius apparatus of HTD-1 type Hitachi, giving a single symmetrical or a single, nearly symmetrical boundary after electrophoresis (Fig. 1). As to the mobilities (See the explanation of Fig. 1), AP II$_2$ and II$_3$ closely resembled AP II$_1$, whereas AP III$_3$ and IV$_3$ differed from them as well as from each other.

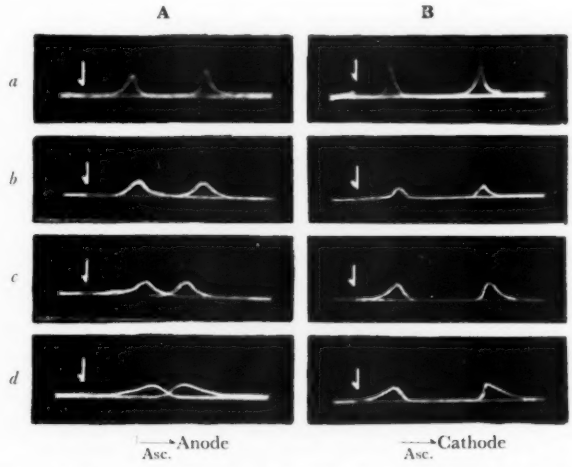

Fig. 1. Electrophoretic patterns ($\times 2/3$) of 1% solutions of AP II$_2$ (*a*), II$_3$ (*b*), III$_3$ (*c*) and IV$_3$ (*d*) in veronal-HCl buffer, pH 8.6, I 0.2 (**A**) and glycine-HCl buffer, pH 2.0, I 0.2 (**B**); current 5 mA; exposure 120 min. after starting current; temperature of test 10°C in **A**-*a*, **A**-*b*, **B**-*a*, **B**-*b* and **B**-*c* and 12°C in **A**-*c*, **A**-*d* and **B**-*d*. Mobility: AP II$_2$, $\frac{(-1.15)\times 0.01043\times 0.18}{120\times 60\times 0.005} = -6.00\times 10^{-5}$ cm^2/volt/sec in the veronal-HCl buffer and $\frac{(+0.75)\times 0.01544\times 0.18}{120\times 60\times 0.005} = +5.99\times 10^{-5}$ cm^2/volt/sec in the glycine-HCl buffer; AP II$_3$, -5.84×10^{-5} cm^2/volt/sec in the veronal-HCl buffer and $+6.18\times 10^{-5}$ cm^2/volt/sec in the glycine-HCl buffer; AP III$_3$, -6.93×10^{-5} cm^2/volt/sec in the veronal-HCl buffer and $+7.95\times 10^{-5}$ cm^2/volt/sec in the glycine-HCl buffer; AP IV$_3$, -8.56×10^{-5} cm^2/volt/sec in the veronal-HCl buffer and $+4.77\times 10^{-5}$ cm^2/volt/sec in the glycine-HCl buffer.

Toxohormone activity. By our usual method,[7] the results illustrated in Table I were obtained. The preparations proved all to be toxohormones, depressing liver-catalase *in vivo* but not *in vitro* and furthermore being ineffective on blood and kidney catalase *in vivo*. The minimum active doses of AP II_2 and II_3 looked to lie between 1 and 3 mg. as that of AP II_1 did[6], but those of AP III_3 and IV_3 were 3 mg.

TABLE I-A

Liver-catalase Depression *in vivo*

The average O_2 volume or the average k_{25} value for each dosis in each test-set is expressed in per cent of the corresponding O_2 volume or k_{25} value in the blank control.

Assay No.	Number of mice used	Amount of substance injected (mg.)		
		5	3	1
By AP II_2:				
1	9	50.0		87.0
2	12	68.6	66.9	91.5
3	6		68.5	
4	6		70.0	
Average		59.3	68.5	89.3
By AP II_3:				
5	12	65.2	79.7	103.3
6	6	64.5		
7	6		71.7	
8	6			91.9
9	6	66.7		
10	6		61.0	
Average		65.5	70.8	97.6
By AP III_3:				
11	12	58.1	74.6	90.3
12	6	60.1		
13	6		75.9	
14	6			92.8
15	6	60.3		
16	6		73.5	
Average		59.5	74.7	91.6
By AP IV_3:				
17	9	74.7		85.8
18	6	63.1		
19	9		73.7	90.6
20	6		74.7	
21	6	64.3		
22	9		69.2	92.1
Average		67.4	72.5	89.5

TABLE I-B

Effect *in vivo* on Kidney- and Blood-catalase

Assay No.	Kidney	Blood	Liver
Effect of AP II_2 (5 mg.) :			
	Oxygen evolved (cc.) in blank experiment		
1	2.2	2.0	10.0
2	3.5	2.6	10.7
3	4.9	2.5	11.4
Average	3.5	2.4	10.7
	Oxygen evolved (cc.) in main experiment		
4	2.9	3.0	7.6
5	2.6	1.9	6.5
6	4.4	2.6	8.4
Average	3.3	2.5	7.5
(% of control value)	(94%)	(104%)	(70%)
Effect of AP II_3 (5 mg.) :			
	Catalase activity (k_{25}) in blank experiment		
7	0.004436	0.003594	0.007772
8	0.004988	0.003908	0.006708
9	0.005248	0.004088	0.006228
Average	0.004891	0.003864	0.006903
	Catalase activity (k_{25}) in main experiment		
10	0.004628	0.004480	0.003580
11	0.004944	0.003752	0.005556
12	0.004436	0.003428	0.004664
Average	0.004669	0.003887	0.004600
(% of control value)	(95%)	(101%)	(67%)
Effect of AP III_3 (5 mg.) :			
	Catalase activity (k_{25}) in blank experiment		
13	0.004124	0.004076	0.005200
14	0.005148	0.002820	0.005428
15	0.003888	0.003016	0.005588
Average	0.004387	0.003304	0.005405
	Catalase activity (k_{25}) in main experiment		
16	0.003508	0.003936	0.003760
17	0.004804	0.002728	0.003120
18	0.005412	0.002912	0.003006
Average	0.004575	0.003192	0.003295
(% of control value)	(104%)	(97%)	(61%)
Effect of AP IV_3 (5 mg.) :			
	Catalase activity (k_{25}) in blank experiment		
19	0.006696	0.003868	0.007564
20	0.006824	0.003724	0.007784
21	0.007452	0.004960	0.006688
Average	0.006991	0.004184	0.007345
	Catalase activity (k_{25}) in main experiment		
22	0.006040	0.003952	0.004652
23	0.007028	0.004420	0.004840
24	0.007276	0.004172	0.004696
Average	0.006781	0.004181	0.004729
(% of control value)	(97%)	(100%)	(64%)

TABLE I-C

Effect *in vitro* on Liver-catalase

Assay No.	Liver-catalase activity (oxygen evolved in cc.)		
	Control I (No incubation)	Control II (Incubated without toxohormone, 20°C, 60 min.)	Main (Incubated with toxohormone, 20°C, 60 min.)
Effect of AP II_2 (5 mg.) :			
1	12.8	9.0	8.3
2	10.2	6.7	7.3
3	9.8	6.0	6.4
Average	10.9	7.2	7.3 (101% of Control II value)
Effect of AP II_3 (5 mg.) :			
4	15.8	12.0	12.9
5	13.7	10.5	11.2
6	10.7	7.6	8.0
Average	13.4	10.0	10.7 (107% of Control II value)
Effect of AP III_3 (5 mg.) :			
7	16.0	13.9	14.8
8	19.0	14.8	15.4
9	20.0	15.2	16.0
Average	18.3	14.6	15.4 (105% of Control II value)
Effect of AP IV_3 (5 mg.) :			
10	17.2	14.2	15.0
11	18.8	14.8	15.5
12	17.8	14.0	14.8
Average	17.9	14.3	15.1 (106% of Control II value)

KIK potency. The procedure described by Masamune and Kawasaki[8] was applied for the determination. As shown in Table II, even 1.5 mg. per kg. body weight of the preparations could not induce 10% decrease of red cell number in rabbits, when injected intravenously, that is, they are KIK inactive.

Test-tube tests. All the preparations behaved similar: biuret ±, Molisch +.

Paper chromatography of sugars and amino acids constituting the preparations. The preparations were treated as AP II_1 and II′ and found to contain the same sugars as those in these preparations, that is, galactosamine, glucosa-

TABLE II

Anemia-inducing Potency

Assay No.	Rabbit No.	Body wt. (kg.)	Sex	Amount of substance injected per kg. body weight (mg.)	Number of red cells ($\times 10^4$, per mm^3) Before inj.	Time after injection (hrs.) 1	2	3	4	5	Maximum decrease (%)
The potency of AP II_2											
1	61	2.7	♀	1.5	624	606	606	600	**572**	581	8
2	53	3.4	♂	1.5	742	747	745	739	762	**737**	1
The potency of AP II_3											
3	49	2.2	♂	1.5	658	661	656	626	**615**	629	7
4	51	2.5	♂	1.5	731	747	717	705	**696**	703	5
The potency of AP III_3											
5	41	3.0	♀	1.5	639	643	648	623	**606**	610	5
6	45	4.0	♀	1.5	503	537	509	493	**469**	477	7
The potency of AP IV_3											
7	46	3.2	♀	1.5	594	605	**577**	583	613	596	3
8	48	2.9	♂	1.5	567	555	559	538	556	**537**	5

mine, galactose and mannose. As to the amino acids, the chromatography of a hydrolysate of the preparations was carried out by a modification of the procedure in the same article that dealt with AP II_1 and II′, letting solvent 80% phenol flow on a square of Toyo Roshi filter paper No. 51 in an atmosphere containing ammonia for the secondary development. Spots of aspartic and glutamic acids, serine, glycine, threonine, alanine, tyrosine, valine and/or methionine, phenylalanine, leucine and/or isoleucine, proline and hexone bases were given by all of AP II_2, II_3, III_3 and IV_3.

Quantitative analyses. The analytical figures obtained are summarized in Table III. Because of their minute contents, quantitative discrepancies of the sugar components, even if present, are discerned neither among the preparations nor between them and AP II_1[6].

Specific refraction. 1% solutions of the preparations (pH 8.0) were examined in an Abbe refractometer at 17°C, finding that all of them have the same specific refraction which is nearly equal with that of AP II_1[6].

$$[n]_D^{17}=\frac{1.33513^2-1}{1.33513^2+2}\times\frac{1}{1.0035}\times\frac{100}{1.000}-\frac{1.33324^2-1}{1.33324^2+2}\times\frac{1}{1.0003}\times\frac{99}{1.000}=0.250$$

TABLE III

Composition in per cent of the Preparations

Analysis	AP II_2	AP II_3	AP III_3	AP IV_3
N*	13.5	13.5	13.2	13.8
Ash†	0.5	0.3	0.3	0.4
Hexose as galactose‡				
PHR_2	1.6	2.2	1.6	1.7
$SMHR_2$	0.5	0.1	0.1	0.1
HR_{10}	2.2	2.7	1.7	1.7
Hexosamine§	1.5	1.1	0.8	0.8
Pentose//	0	0	0	0
Methylpentose¶	0	0	0	0
Sialic acid**				
ER	0	0	0	0
DR	0	0	0	0

* Micro Kjeldhal method. † Pregl method without use of H_2SO_4. ‡ Masamune and Sakamoto[9]. § Masamune and Yosizawa[10]. // Masamune and Sakamoto[11]. ¶ Masamune and Kawasaki[12]. ** Werner and Odin (direct Ehrlich method and diphenylamine method)[13].

DISCUSSION

Masamune, Kaketa and S. Abe[5] claimed that the corresponding toxohormones from the same source of cancer patients with different sites of original tumor are identical, as mentioned above. In conformity with that opinion, AP II_1, II_2 and II_3 the corresponding protein components of the cancerous ascitic fluids from a gastric cancer-, a liver-cancer- and an ovarian cancer-patient have been proved identical from the findings regarding electrophoretic mobility, strength of toxohormone activity, composition and optical refraction.

AP II_3, III_3 and IV_3 which vary in electrophoretic mobility, in precipitability with ammonium sulfate, in toxohormone activity and in solubility in water (II_3 and III_3 are similar in solubility, and III_3 and IV_3 in toxohormone activity) must be different chemical entities.

CONCLUSIONS

1. Toxohormones AP II_1, II_2 and II_3 which are the protein fraction precipitating at 1.0–1.4 *M* of ammonium sulfate and at 40–60% of ethanol of cancerous ascitic fluids from patients with original tumor in stomach, liver or ovary are identical with one another.

2. AP III_3 and IV_3 which were protein fractions, one precipitating at 1.6–2.0 *M* ammonium sulfate and the other precipitating at 2.4–2.8

M concentration of the same salt, of the ascitic fluid from an ovarian cancer patient differed from each other and also from AP II_3 from the same source.

The expenses of this work were defrayed by a grant given from the Ministry of Education through the Grant Committee for Medical, Dental and Pharmaceutical Researches. H. Masamune.

References and Notes

1) Masamune, Kawasaki & Abe, S., Tohoku J. Exp. Med., 1958, **67**, 140.
2) Masamune, Kaketa & Abe, S., *ibid.*, 1958, **67**, 329.
3) Kawasaki, *ibid.*, 1958, **68**, 119.
4) Kawasaki, *ibid.*, 1959, **69**, 153.
5) Masamune, Kaketa & Abe, S., *ibid.*, 1958, **68**, 321.
6) Masamune, Kamiyama, Abe, S., Abe, Sh., Haga & Kaketa, *ibid.*, 1959, **69**, 245.
7) Masamune, Tsuiki, Kamiyama, Abe, S., Haga, Kawasaki, Kaketa & Abe, Sh., *ibid.*, 1958, **67**, 309.
8) Masamune & Kawasaki, *ibid.*, 1956, **63**, 369 (f. 372).
9) Masamune & Sakamoto, *ibid.*, 1956, **63**, 345.
10) Masamune & Yosizawa, *ibid.*, 1957, **65**, 169.
11) Masamune & Sakamoto, *ibid.*, 1956, **63**, 357.
12) Masamune & Kawasaki, *ibid.*, 1956, **63**, 381 (f. 382).
13) Werner & Odin, Acta soc. med. Upsal., 1952, **57**, 270.

The Tohoku Journal of Experimental Medicine, Vol. 69, Nos. 2-3, 1959

The Studies on the Kidney Homotransplantation

III. Immunological Induction of Similar Histological Pattern of the Kidney as That of Homotransplanted Kidney

By

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(Received for publication, January 16, 1959)

It has been confirmed by our previous studies that, some histological changes which appeared in the homotransplanted kidney could be observed in the recipient kidney which had not been removed.[1] Moreover, the same histological changes as that found in the homotransplanted kidney could be produced in the kidney by the homologous cross-circulation.[2] In the present study, we could induce the above histological changes in the kidney of dog which had been immunized by the kidney preparation from another dog as an antigen. The liver of immunized animals also showed similar changes as in the case of homologous transplantation of the kidney.

Experimental

Methods

Antigen. Healthy adult dogs were used. Directly after the removal, organs were irrigated with physiological saline solution to remove the contamination of blood. Capsules were stripped off (in the case of kidney, cortex only was used after removal of medulla), chopped, and washed with distilled water for 24 hours, and then homogenized. Antigen was prepared from these tissue homogenate as follows; 25 g. of 60 per cent of the homogenate was added with 25 g. of lanolin, 100 g. of paraffin and 100 mg. of dried tubercle bacilli of Aoyama B-strain as adjuvant[3),4),5)].

Immunization. Healthy adult dogs were injected subcutaneously with the antigen (3–4 m*l.*/kg.) every three or four days for several weeks.

Histological examinations. Specimens were prepared as follows: soon after experimental animals died or they were killed by the intravenous injection of pentobarbital, organs were removed and fixed with 10 per cent formalin solution, stained with hematoxilin-eosin, van Gieson's method

methylgreen-pyronin (Unna-Papenheim staining). On these specimens, histological changes were observed microscopically.

Results

Experiment I (*One healthy dog was immunized with the kidney preparation from another healthy adult dog*) (Table I). Seven animals were subjected to this

TABLE I

One Healthy Dog was Immunized with the Kidney Preparation from Another Healthy Adult Dog

Exp. No.	Body weight (kg.)	Dose (ml.)	No. of injection		Histological findings
1	3	8	8	killed	Interstitial nephritis accompanied by pyroninophilic cell infiltration
2	4	8	13	"	"
3	4	10	15	"	"
4	3.5	10	15	"	"
5	3	8	10	"	"
6	3	8	6	died	
7	3	8	5	"	

experiment. Of these, two animals (No. 6 and 7) were died from malnutrition, and pyogenic change was observed at the site of inejction performed, so that histological examinations were not carried out with them.

Each of the remaining animals was injected with the antigen, eight, ten, thirteen or fifteen times, respectively. These were sacrificed and histological changes were observed as discribed above. The kidney of each experimental animal showed common histological changes. Thus, dilatation of the capillaries and the proliferative changes of endothelial cells were observed in the glomeruli. The tubules dilated slightly, and nuclei had been disappeared in some of the tubular cells. Moreover, infiltration of round cells and plasma cells could be observed especially around the blood vessels in the interstitial space, and the most of these cells were pyroninophilic. Similar changes could be found around the glomeruli. Although the degree of the changes was generally slighter than what observed in the homotransplanted kidney, this could evidently be recognized as interstitial nephritis accompanied with infiltration of pyroninophilic cells (Fig. 1 and 2).

Histological changes observed in the liver of immunized animals were more interesting. Thus, in two cases (No. 3 and No. 4), pyroninophilic round cell infiltration could be observed around the blood vessels of the

Glison's capsule. These changes were quite similar as that found in the recipient liver after the homologous transplantation of the kidney (Fig. 3). Details will be discussed below.

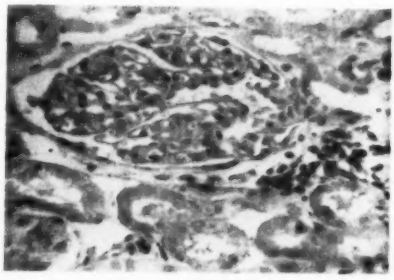

Fig. 1. Exp. No. 3. M-P staining. ×300. Showing periglomerular accumulation of round cells including many pyroninophilic cells.

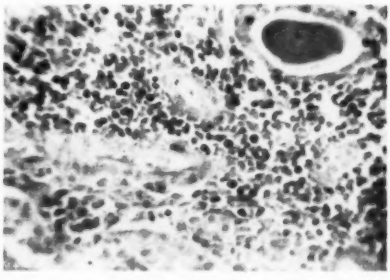

Fig. 2. Exp. No. 3. M-P staining. ×300. Showing perivascular accumulation of pyroninophilc cells in the interstitial space.

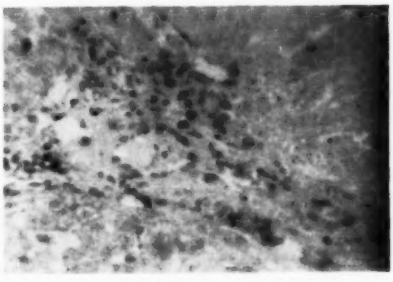

Fig. 3. Exp. No. 3. M-P staining. ×300. Showing pyroninophilic round cells infiltration around the blood vessels of Glison's capsule.

In this experiment, the same histological pattern as in the case of kidney homotransplantation could be induced not only in the kidney but

also in the liver of dog which had been immunized with a suspension of kidney tissue from another dog.

Experiment II (*One healthy dog was immunized with the kidney preparation from several individuals*) (Table II). This experiment was carried out on five cases (No. 8 to 12), and four of them (No. 9, 10, 11 and 12) were subjected

TABLE II

One Healthy Dog was Immunized with the Kidney Preparation from Serveal Individuals

Exp. No.	Body weight (kg.)	Dose (*ml.*)	No. of injection		Histological findings	
8	3	8	5	died		
9	3	10	10	killed	Interstitial nephritis	(−)
					Degeneration of liver	(+)
10	3.5	10	10	"	"	(−)
					"	(+)
11	4	10	8	"	"	(−)
					"	(+)
12	3.5	10	8	"	"	(−)
					"	(+)

to microscopical examinations. In these cases, however, neither interstitial nephritis nor pyroninophilic cells were observed.

In the kidney, though dilatative change of the glomerular capillaries was not be found, endothelial cells were proliferated slightly and hyperemia was also observed. There was no special finding in the tubular cells and no cell infiltration was found in the interstitial space.

The liver showed more pronounced changes; liver cells were dissociated, and polynuclear leucocytes were found in the Disse's canal, and dilatation of sinusoid was also observed. There was no cell infiltration in the Glison's capsule. Liver cells showed no significant changes, but granular- or vacular-degeneration and degenerative necrosis were observed in some parts.

Experiment III (*One healthy dog was immunized with the liver or liver-spleen preparation from another dog.* (Table III). Four aimals (No. 13, 14, 15 and 16) were immunized with the equal volume mixture of liver and spleen suspensions. In this case, experimental animals became anorexic and caused emesis, gradually became asthenic, and died, so that injections could not be performed more than three times. Marked degenerative changes were observed in the liver cells, but interstitial nephritis could not be found in the kidney.

Three animals (No. 17, 18 and 19) were immunized only with the liver tissue preparation. These animals also lapsed into anorexia, mal-

TABLE III

One Healthy Dog Was Immunized with the Liver or Liver-spleen Preparation from Another Dog

Exp. No.	Body weight (kg.)	Dose (ml.)	No. of injection		Histological findings	
13	2.5	8	3	died	Interstitial nephritis	(−)
					Degeneration of liver	(+)
14	3	10	3	"	"	(−)
					"	(+)
15	3	10	3	"	"	(−)
					"	(+)
16	3.5	10	2	"	"	(−)
					"	(+)
17	3.5	10	7	killed	"	(−)
					"	(+)
18	3	10	7	"	"	(−)
					"	(+)
19	3	10	6	died	"	(−)
					"	(+)

nutrition and loss of body weight so that injections could not be continued for a long period of time.

In the kidney, glomerular cell nuclei increased in their number, but hyperemia was not so significant. Exudation was found in Bowman's capsule in some cases. Some of the tubular epithelial cell nuclei had disappeared. Cell infiltration was not evident in the interstitial space except one case (No. 17), in which round cell infiltration and polynuclear leucocytes infiltration could be observed all over the kidney. However, any of these cells was not pyroninophilic.

In the liver, granular- or vacular-degeneration and degenerative necrosis of the liver cells were generally observed. No infection of bacilli was confirmed by the cultivation of blood from two experimental animals (No. 17 and No. 19).

TABLE IV

Animals were Injected with Adjuvant Only as a Control

Exp. No.	Body weight (kg.)	Dose (ml.)	No. of injection		Histological findings
20	2.5	8	10	killed	No special finding in the kidney and the liver
21	3	10	10	"	"
22	3	10	10	"	"

Experiment IV (*Animals were injected with adjuvant only as a control*) (Table IV). Three cases (No. 20, 21 and 22) were examined. No significant change was found except increment of glomerular cell nuclei and dilatation of the tubule. Pyroninoplilic cell could not be found. In the liver, significant changes was not observed.

Discussion

Histological characteristic appeared in the homotransplanted kidney is interstitial nephritis ; especially pyroninophiric round cell-, plasma cell-, or histocytes-infiltration around the capillary blood vessels in the interstitial space and glomeruli are considered as the most distinct character[6),7)].

It has been reported by several authors that pyronin-positive finding by the methylgreen-pyronin staining suggests the existence of ribonucleic acid in the cells[8)]. Ehrlich discovered a corelation between the production of antibody and ribonucleic acid content after the injection of typhoid vaccine into rabbit, and suggested that ribonucleic acid may play an important part in the antibody production[9)].

Besides, a number of workers have been carried out on the basis of an assumption that the histological changes appeared in homotransplanted kidney after the homologous transplantation of kidney may be caused by some immunological reactions.

Thus, some workers attempted to solve the problem from a hypothesis of passive transfer by serum, blood, or spleen extract, etc.[6)]. An attempt was made to estimate the cyto-toxic antibody by serological examination after the homotransplantation of the kidney[11),12),13)]. Another attempt was made to induce an analogous histological pattern by immunological treatment or by cross-circulation[10)].

In our previous reports, it was confirmed that, after the homologous transplantation of the kidney of dog, if recipient kidney was not removed, the same histological changes could be observed in the recipient kidney as in the transplanted kidney, and that the liver of recipient also showed a similar histological changes. From these results, it may be suggested that recipient may be exposed to the same immunological condition, mediated through the transplanted kidney, to which homotransplanted kidney may also be exposed after the transplantation. Successful result was also obtained by the homologous cross-circulation[2)].

In the present experiments, quite similar histological pattern could be induced into the kidney by immunizing one healthy adult dog with the suspension of kidney (added with adjuvant) from another dog as in the case of homotransplantation of the kidney, though the degree of changes was slighter than that in the latter. The liver also showed some histological changes which appeared after the homotransplantation of the kidney.

However, when kidney from more than one individual, liver and spleen from one individual, or liver tissue from one individual was subjected to the antigen, no significant change was observed histologically.

Summary

Similar histological changes as appeared in the homotransplanted kidney could be induced by immunological treatment. That is, the kidney of dog which had been injected hypodermically, an antigen, with the suspension of kidney tissue (added with adjuvant) from another dog showed the same histological pattern as that of homotransplanted kidney.

It is my pleasure to acknowledge the encouragement and advice I have received from Prof. K. Akazaki.

References

1) Ishikawa, Tohoku J. Exp. Med., 1957, **67**, 11.
2) Ishikawa, *ibid.*, 1957, **67**, 19.
3) Freund, Annual Review Microbiol., 1947, **1**, 291.
4) Morgan, J. Exp. Med., 1947, **85**, 131.
5) Kabat, *ibid.*, 1947, **85**, 117.
6) Dempster, Brit. J. Surg., 1953, **40**, 441.
7) Simonsen, Acta Path. Microb. Scand., 1953, **32**. 1.
8) Brachet, J. Biol. Chem., 1940, **135**, 88.
9) Ehrlich, J. Exp. Med., 1949, **90**, 157.
10) Egdahl, Surg. Gynec. Obstet., 1956, **102**, 450.
11) Bollag, Experientia, 1956, **12**, 57.
12) Favour, Proc. Soc. Exp. Biol. Med., 1953, **83**, 352.
13) Muirhead, Arch. Path., 1955, **59**, 223.

The Tohoku Journal of Experimental Medicine, Vol. 69, Nos. 2-3, 1959

Studies on Cirrhosis of the Liver

VII Hepatic Venography in Clinical Cases

By

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For diagnosing liver diseases, various function tests and biopsy of the liver are routinely carried out besides the examinations of clinical signs and symptoms. Recently, in addition, the measurements of hepatic blood flow and of wedged hepatic venous pressure and hepatic venography by means of liver catheterization have come into importance.

The present authors subjected normal subjects and patients with various liver diseases to hepatic venography and studied the morphological changes and anastomoses in their hepatic veins, as reported in the following study.

Experimental

Subjects and Method

Hepatic venography was performed to 11 cases with no known liver disorder (in 3 cases of which the catheter was driven in deeply to introduce the contrast medium into the sinusoids), 4 hepatitis cases, 6 liver fibrosis cases, 11 liver cirrhosis cases comprising 4 mild, 6 severe and one biliary cases, and 3 metastatic liver cancer cases, 35 cases in total.

In these cases, a catheter was inserted through the ulnar or radial vein into the right hepatic vein under X-ray control after the method adopted in 1953 by Ueda[1] and Tori[2]. The tip of the catheter was led as near as possible to the orifice of the right hepatic vein into the inferior caval vein, and about 15 cc of 70% Pyraceton or 60% Urographin was introduced as quickly as possible and a venogram was taken. For taking the venogram of the sinusoids the catheter was driven in more deeply. For testing supersensitivity to iodine, 1–2 cc of contrast medium was

slowly injected intravenously before the venography. Only one case vomited during the test, while the other cases merely complained of a temporary feeling of fever.

Results

1) Changes in the hepatic veins

In the normal liver, the hepatic veins sent out many branches at regular intervals, tapered off gradiually and did not run crooked or serpentine courses. Their walls were even and smooth (Figs. 1 and 2). In the cases with hepatitis and liver fibrosis, no change was observed; in only one of the cases with mild liver cirrhosis, a crooking was found in the course of the veins (Fig. 3). In the severe cirrhosis cases, however, the branches were very few and the veins ran zig-zag courses, giving the impression of a dead tree, as shown in Figs. 4, 5 and 6, while the venous walls were found uneven due to the pressure of the regenerating nodules. The case of biliary cirrhosis, though severe, showed little change (Fig. 7). In

Fig. 1

Fig. 2

Fig. 1. The hepatic veins in the normal liver. A: Anastomoses between the hepatic veins.

Fig. 2. The hepatic veins in the normal liver. A: Anastomoses between the hepatic veins.

the mild liver cancer cases, the branches were somewhat decreased and the courses of the veins were winding. In the severe liver cancer case, the picture of venous stenosis and circuitous courses was apparent, and a round

Fig. 3

Fig. 4

Fig. 3. The hepatic veins in hepatitis. A: Anastomoses between the hepatic veins.

Fig. 4. The hepatic veins in liver cirrhosis.

shadow as shown in Fig. 8 was observed. Upon autopsy of this case, it was ascertained by venography and casting, that the venous walls were infiltrated by the cancerous tissue, probably forming a venous cyst.

In the normal cases with the catheter driven far into the sinusoids, a uniformly dense localized shadow was seen, and the contrast medium running out of it delinated the branches of the portal vein (Fig. 9).

2) Venous Anastomoses

Figs. 10 and 11 illustrating the liver of a hepatitis case showed a picture of anastomoses between the hepatic veins. The anastomoses were clearly demonstrated, as the contrast medium, alternately introduced into the V. hepatica dextra ventralis and the V. hepatica dextra caudalis, as nominated by Toyoshima[3], obviously ran through the anastomoses to the other vein branch.

Anastomoses between the hepatic veins were frequently met with in 7 of 8 normal cases, in 2 of 4 hepatitis cases, in 3 of 6 liver fibrosis cases and in 3 of the 4 mild liver cirrhosis cases, but only in one of the 6 severe cirrhosis cases and in one biliary cirrhosis case. In the cases of severe liver cancer, besides the anastomoses usually observed in normal cases, complicated anastomoses between hepatic venous branches resembling of meshes of a

Fig. 5. The hepatic veins in liver cirrhosis.

Fig. 6. The hepatic veins in liver cirrhosis.

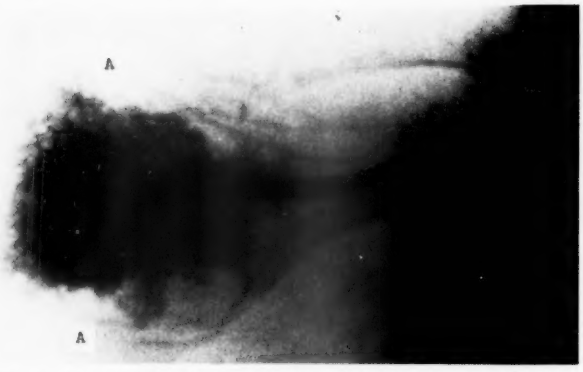

Fig. 7. The hepatic veins in biliary liver cirrhosis. A : Anastomoses betweentween the hepatic veins.

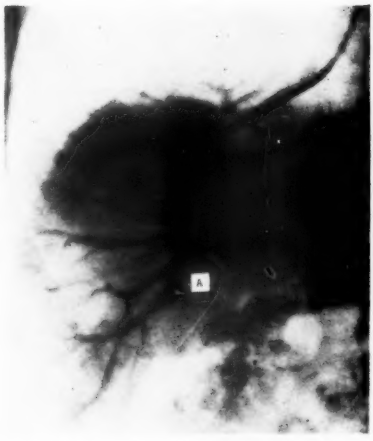

Fig. 8. The hepatic veins in metastatic liver cancer. A : Abnormal anastomoses between the hepatic veins. B : Anastomoses between the hepatic veins. C : Round shadow.

Fig. 9. The contrast medium is injected into sinusoids in the normal liver. A : Portal veins.

Fig. 10. The contrast medium flows from the V. hepatica dextra ventralis into the V. hepatica dextra caudalis through the anastomosis. A : Anastomosis.

net were observed.

As described in the succeeding paper, in the autopsied cases of liver

Fig. 11. The same liver as shown in Fig. 10. The contrast medium flows from the V. hepatica dextra caudalis into the V. hepatica dextra ventralis through the anastomosis. A : Anastomosis.

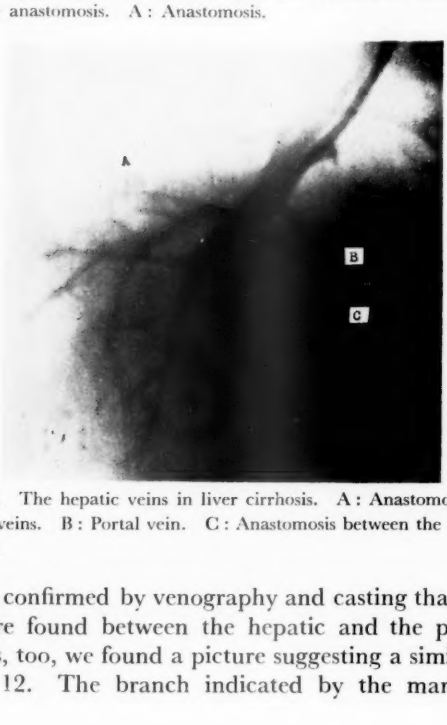

Fig. 12. The hepatic veins in liver cirrhosis. A : Anastomosis between the hepatic veins. B : Portal vein. C : Anastomosis between the hepatic and portal veins.

cirrhosis, it was confirmed by venography and casting that relatively large anastomoses were found between the hepatic and the portal veins. In our clinical cases, too, we found a picture suggesting a similar anastomosis, shown in Fig. 12. The branch indicated by the mark "c" in the

figure can hardly be a branch of the right hepatic vein, judging from its course, but seems to be a branch of the portal vein.

Discussion

Since Rappaport[4] employed hepatic venography to dogs, there have been several reports on the subject. In the normal liver, Tori[2] reported the satisfactory visualization of the right hepatic veins as far as their terminal branches, and Takahashi[5] and Ney[6] asserted that the course of the hepatic veins was never rugged, and the branches of the veins tapered off slowly and were large in number. In the cases in which contrast medium was introduced through the sinusoids, Celis[7] reported a homogenous shadow corresponding to the opacified vascular territory. In 4 cases with liver cirrhosis, to which venography was applied by Takahashi[5], venous branches were reduced in number and as knotty as plum tree branches. In liver tumor, Tori[2] found the number of branches reduced and Mikami[8] observed absence of the shadows and a stricture due to pressure and displacement of the hepatic veins. All the above observations have been confirmed in our studies.

In hepatitis, liver fibrosis, and mild liver cirrhosis cases little change in the venous picture has been found in the present studies, in close agreement with the remarks of Wakim[56] that little change was found in the autopsied cases of early liver cirrhosis.

Anastomoses between the hepatic veins were observed in most of the autopsied cases of normal livers and in most of our clinical cases. Although Ney[6] considered that the same anastomoses as shown in Figs. 11 and 12, were the anastomoses between portal and hepatic veins, it is clear from the two photographs that they represent the anastomoses between the hepatic veins.

The existence of anastomoses between the hepatic and the portal veins in liver cirrhosis cases is proved by what we report on autopsied cases is in the succeeding paper, but no such anastomosis observed venographically in clinical cases has previously been reported. The present authors have experienced a picture suggesting the anastomosis between the hepatic and the portal veins in one of our clinical cases of liver cirrhosis.

Summary

Using liver catheters, we employed hepatic venography to study normal subjects and clinical patients with liver diseases and arrived at the following results in summary:

1. Little change from the normal was observed in the hepatic veins in

hepatitis, liver fibrosis and mild liver cirrhosis, but in severe liver cirrhosis, the branches were fewer, as if it were a dead trees, showing marked changes in the general picture. In the case of severe but biliary cirrhosis, however, no change was observed. In the cases of liver cancer, circuitous courses of the hepatic veins and a round shadow were observed.

2. When the contrast medium was introduced as far as possible into the sinusoids, the sinusoids as well as the portal vein branches also came out in the venograms.

3. Anastomoses between the hepatic veins were observed in most of the normal livers and we could observe similar anastomoses in hepatitis, but they were found rarely in liver cirrhosis.

4. A finding suggesting anastomoisis between the hepatic and the portal veins was observed in a liver cirrhosis case.

References

1) Ueda, Nihon Naikagakkai Zasshi (Jap.), 1953, **42**, 591.
2) Tori, Acta radiologica, 1953, **39**, 87.
3) Toyoshima, Nihon Geka Hokan (Jap.), 1954, **23**, 476.
4) Rappaport, Acta radiologica, 1951, **36**, 165.
5) Takahashi, Tokyo Jikeikai Idai Zasshi (Jap.), 1954, **69**, 708.
6) Ney, Fortschr. Röntgenstr., 1957, **86**, 302.
7) Celis, Villalobos, Castillo & Espinosa, Am. J. Roentgen., 1955, **74**, 1089.
8) Mikami, Nihon Gekagakkai Zasshi (Jap.), 1956, **57**, 898.
9) Wakim, Proc. Staff Meet. Mayo Clin., 1953, **28**, 218.

The Tohoku Journal of Experimental Medicine, Vol. 69, Nos. 2-3, 1959

Studies on Cirrhosis of the Liver

VIII Angiograms and Casts of Autopsied Livers

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(Received for publication, January 21, 1959)

In our previous study[1] we employed venography to study the liver of normal subjects and patients with liver diseases and obtained results that may contribute to better diagnosis of such diseases. In the present study, we employed hepatic angiography and casting to livers of autopsied cases with and without liver diseases including cirrhosis, for studying the alterations and the anastomoses in their blood vessels.

Experimental

Materials and Method

Our study was conducted on 11 normal livers, 1 liver with fibrosis, 5 livers with cirrhosis and 3 livers with metastatic cancer, 20 livers in total.

First, vinyl tubes were introduced into the hepatic veins, the hepatic artery and the portal vein. Then the material was flushed with physiological saline and 50% potassium iodide solution was infused into the vessels and angiography was performed. After this, vinyl acetate coloured blue, red and white was infused into the hepatic veins, the portal vein and the hepatic artery, respectively, and after its hardening by polymerisation, the tissues were corroded away with hydrochloric acid, and the cast was taken out for macroscopic examination.

Results

1) Vascular changes

In normal livers, the hepatic veins were found, as in the clinical cases, sending out numerous branches, tapering off steadily but slowly, and showing neither crooking in their courses nor flattening by pressure

of their walls (Fig. 1). In liver fibrosis and mild liver cirrhosis, the vessels were little changed. In severe liver cirrhosis, the hepatic veins were found the most heavily changed of all vessels, namely, they became thin during

Fig. 1. The hepatic veins in the normal liver. A : Anastomoses between the hepatic veins.

their courses which were severely crooked and distorted and were flattened and quite obstructed in some extreme cases by pressure of the proliferating nodules. Their branches were extremely reduced in number (Figs. 2, 3 and 4). In the liver with metastatic cancer, the areas drained by the

Fig. 2. The hepatic veins in liver cirrhosis.

hepatic veins were smaller and their branches were fewer, while the vessels

Fig. 3

Fig. 4

Fig. 3. The hepatic veins in liver cirrhosis.
Fig. 4. The cast of the hepatic veins in liver cirrhosis.

were irregularly displaced to avoid the metastatic foci of cancer. The round shadow observed in a clinical case, upon autopsy, was seemingly found due to a vascular cyst formed by deciduated tissues (Figs. 5 and 6).

Fig. 5. The hepatic veins in metastatic liver cancer. A : Anastomoses between the hepatic and portal veins.

The portal vein sent out branches radially and had a smooth wall in

Fig. 6. The cast of metastatic liver cancer. Dark-hepatic vein, white-hepatic artery. A: Round shadow.

Fig. 7. The portal veins in the normal liver.

normal livers (Fig. 7). In the case of liver fibrosis, some branches of the portal vein were found running spiral courses. In severe liver cirrhosis, the vein was found to run crooked or serpentine courses, though not so badly disfigured as the courses of the hepatic veins. In some cases, several spiral branches of the portal vein were found in groups (Fig. 8). In metastatic liver cancer, the portal vein was displaced, its course deranged, its branches decreased, and the metastatic foci were circumvented (Fig. 9).

Fig. 8. The portal veins in liver cirrhosis. A: Anastomoses between the portal veins.

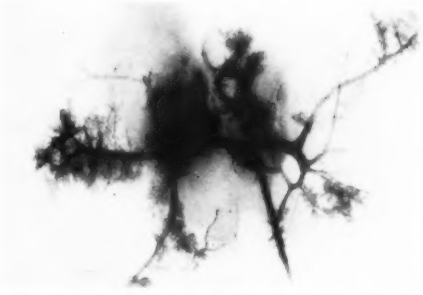

Fig. 9. The portal veins in metastatic liver cancer. A: Anastomosis between the hepatic and portal veins. B: Anastomoses between the portal veins.

The hepatic artery ran along the portal vein in the normal liver. In liver cirrhosis, the arterial branches were found increased (Fig. 10). In metastatic liver cancer, it was also overgrown, and its major branches kept off the metastatic foci but its minor branches aggregated around the foci and entered a short way into them (Figs. 11 and 12).

2) Vascular Anastomoses

Anastomoses between the hepatic veins were found in 10 of the 11 normal cases, so that such anastomoses may be called usual in normal livers. The presence of such anastomoses was proved by the transition of the contrast medium through the anastomotic junctions and it was evidenced in the casts of the same specimens (Figs. 13 and 14). The same anastomoses were found in liver fibrosis and mild liver cirrhosis, too, but in none of the 4 cases of severe cirrhosis. In metastatic liver cancer, besides the abovenamed anastomoses, we met with some of apparently pathological nature,

Fig. 10. The hepatic arteries in the normal liver.

Fig. 11. The hepatic arteries in metastatic liver cancer.

namely, anastomoses between the veins cut by invasion of cancerous tissue and neighboring venous branches and mesh-like anastomoses in the vicinity of the metastatic foci (Fig. 15).

Anastomoses between the portal veins were discovered in 2 of 7 normal livers. In the cancerous liver, similar anastomoses were observable, but in cirrhotic livers, no such normal anastomoses were present, but instead seemingly pathological anastomoses between portal branches in spiral form were observed.

Anastomoses between the hepatic arteries were observed in only 2 of the 6 normal livers and never otherwise.

The shunt, i.e., the anastomoses between the hepatic veins and the portal veins coming into question in liver cirrhosis were found in one of

Fig. 12. The cast of metastatic liver cancer. White-hepatic artery, dark-hepatic vein and grey-portal vein.

the severely cirrhotic livers, but never in normal, fibrotic, or mildly cirrhotic livers. Figs. 16, 17 and 18 show the hepatic and the portal venograms and the casts of the vascular systems of the severely cirrhotic liver with such anastomoses, clearly showing that the contrast medium introduced into the hepatic veins have passed into the portal veins. The portal branches in Fig. 16 containing the contrast medium coming over from the hepatic veins were the same as those shown in Fig. 17, while in Fig. 18, we see the anastomoses between the hepatic veins and the portal veins, causing a mixture of the blue and the red synthetic resin. In metastatic liver cancer, such anastomosis was observed (Figs. 8 and 9). In the extreme case, the vascular tissue was destroyed to form a vascular cyst, as indicated by the above-mentioned round shadow and the heaptic and the portal veins coming into anastomosis by coming together in the cyst.

Anastomosis between the hepatic vein and artery or between the portal vein and the hepatic artery was not noticeable by our methods in any of our cases.

Discussion

Almost all authors are in agreement on the changes in the vascular systems in liver diseases. In the case of liver cirrhosis, Kretz[2] said that

Fig. 13. Anastomoses between the hepatic veins in the normal liver.
A : Anastomosis.

the veins were distorted, McIndoe[3] that the venous branches were perceptibly reduced in number, the larger of them being twisted about, and Takahashi[4] reported on observations similar to ours. Wakim[5] said that no change was observed in the veins of early cirrhotic livers, and no change was found in the mild cases we studied either. On the artery in liver cirrhosis, McIndoe[3] asserted its atrophy while Kretz[2] remarked that it was dilatated and increased. Madden[6] emphasised the fact that the vascular changes were dissimilar in cases with and without ascites, the venous branches decreasing and the portal and the arterial branches increasing in the cases with irreversible ascites, but all the branches universally decreasing in the cases without, or with only reversible, ascites. The above-mentioned difference of the findings by McIndoe[3] and Kretz[2] may be interpreted as due to such difference in the behavior of the blood vessels by cases. We also found the arterial branches increased in a case with irreversible ascites. In liver cancer, Wakim[5] as well as Madden[6] said that the arterial branches were increased for better nourishment of the cancerous tissue. We also found these increased.

Fig. 14. The cast of the hepatic veins in the normal liver. A : Anastomoses between the hepatic veins.

While the reports on the vascular changes are thus in nearly complete accord, those on vascular anastomoses in the liver are not so concordant, seemingly owing to the different results obtained by different experimental methods.

Elias[7] and some others have admitted existence of anastomoses between the hepatic veins and their branches in many cases, and Mann[8] only between the minor branches, in normal livers. We have found anastomoses of 1 mm. or above in diameter in most of the normal livers. It is probable that microscopic anastomoses come forth in considerable number in such livers.

Anastomoses between the portal veins were labeled as rare by Mann[8] and others and Elias[7] and Popper[9] also reported them as few in occurrence. We have found them in about a third of the normal livers. In liver cirrhosis, Popper[9] found them rather frequently. We found some cases of spiral anastomoses of the branches.

Anastomoses between the hepatic arteries were affirmed by Mann[8] among its minor branches in only a few cases. We found such in a third of our cases.

Anastomoses between the hepatic and the portal veins were denied by Popper[9], Mann[8] and Elias[7] in normal livers and we also failed to find

Fig. 15. Abnormal anastomoses between the hepatic veins in the cast of metastatic liver cancer.

them, but Madden affirmed their existence.

In cirrhotic livers, Mann[8] fairly commonly found anastomoses of fair size between hepatic and portal vains by vinylite injection cast method. According to Popper[9], however, anastomoses between hepatic and portal veins were visible only in the vicinity of the coronary and falciform ligaments by vinylite injection cast method, whereas porto-hepatic venous anastomoses in size from 10 to 70 microns in diameter were frequently observed in the cirrhotic livers injected with colored gelatin and india ink. In the present investigation, which were performed by the method of synthetic resin casting and venography, the shunts from 1 to 2 mm. in diameter were demonstrated in one case with liver cirrhosis, as shown in Figs. 16, 17 and 18. Of course, there may be the porto-hepatic venous anastomoses of smaller size, which can be demonstrated only microscopically, in the other cirrhotic livers, although we could not confirm by the cast method.

Anastomoses between the hepatic veins and arteries were denied by Madden[6] in normal and by Mafadzean[10] in cirrhotic livers. Neither could we find such cases.

In normal livers, anastomoses between the portal veins and the hepatic arteries were not found by Mann[8] and Popper[9], and confirmed by Wakim[5] only among the minor branches. However Mafadzean[10] admitted their existence in cirrhosis. Not one of such case was found by our methods

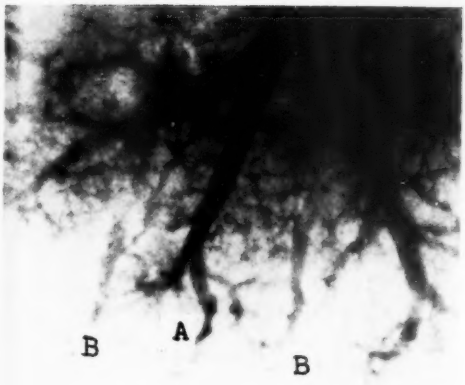

Fig. 16. The hepatic venogram in liver cirrhosis. A: Hepatic vein. B: Portal vein.

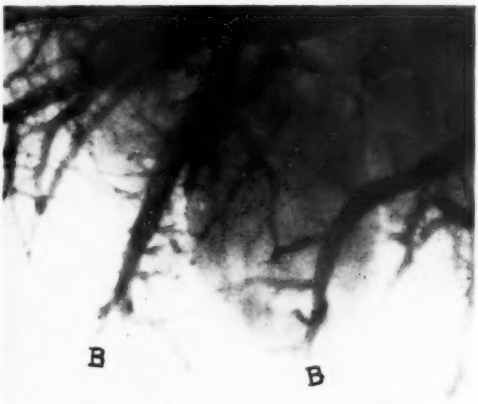

Fig. 17. The portal venogram in liver cirrhosis. B: Portal vein.

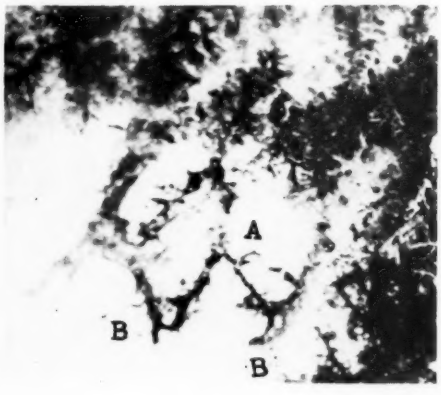

Fig. 18. The cast of liver cirrhosis. Dark-hepatic vein, grey-portal vein. A: Hepatic vein. B: Portal vein.

among our subjects.

Summary

For obtaining data endorsing what we have reported previously on the results of hepatic venography in clinical cases, we employed venography and vascular casting on livers in autopsied cases of normal and diseased livers. The results were well reconcilable with those of the previous study, as summarized in the following:

1. In the severely cirrhotic livers, the changes were most prominent in the hepatic veins, which showed the distorted appearance of dead trees. In mild cirrhosis, almost no vascular change was observed. In cancerous livers, the veins were found displaced and circuitous, their branches being reduced in number. In a severe case, a vascular cyst was found in formation.

2. The portal vein was found less affected than the hepatic veins in cirrhotic livers, but some of its branches were found running spirally.

3. The hepatic arterial branches were augmented in the cirrhotic cases with ascites, and also in the cancerous livers but not penetrating deep into the foci.

4. Anastomoses between the hepatic veins were observed in most of the normal livers but not in the severely cirrhotic livers. In a case of cancerous liver, we found pathological mesh-like anastomoses.

5. In the severely cirrhotic livers, anastomoses between the hepatic and portal veins were found to exist. In a cancerous liver, the necrotic tissue had formed a vascular cyst and the hepatic and the portal veins were found anastomosed within it.

References

1) Nakamura, T., Nakamura, S., Watanabe, Suzuki, Tokita, Kimura, Sato & Kaneko, Tohoku J. Exper. Med., in press.
2) Kretz, Internat. Clin., 1905, **3**, 289.
3) McIndoe, Arch. Path., 1928, **5**, 23.
4) Takahashi, Tokyo Jikeikai Idai Zasshi (Jap.), 1954, **69**, 708.
5) Wakim, Proc. Staff Meet. Mayo Clin., 1953, **28**, 218.
6) Madden, Loré, Gerold & Rapid, Surg. Gyn. Obst., 1954, **99**, 385.
7) Elias & Petty, Am. J. Anat., 1952, **90**, 59.
8) Mann, Wakim & Baggenstoss, Proc. Staff Meet. Mayo Clin., 1953, **28**, 227.
9) Popper, Elias & Petty, Am. J. Clin. Path., 1952, **22**, 717.
10) Mafadzean & Gray, Lancet, 1953, **265**, 1128.

The Tohoku Journal of Experimental Medicine, Vol. 69, Nos. 2-3, 1959

Approximate Estimation of Tuberculin-Polysaccharide in Serum

(Studies on Tuberculosis in Infancy and Childhood 4th Report)

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(Received for publication, January 22, 1959)

It was reported by us[1] that when tuberculin-polysaccharide was added to human blood in vitro, it was firstly combined with the antibody against the tuberculin-polysaccharide in the serum, and then, when the amount of tuberculin-polysaccharide added was too large for that of the antibody, the tuberculin-polysaccharide in excess was adsorbed to the erythrocytes. In other words, when the erythrocytes sensitized with tuberculin-polysaccharide were demonstrated[2] in the circulating blood, it was either the case in which too large an amount of tuberculin-polysaccharide had been produced from tuberculous foci or the case in which the antibody against the tuberculin-polysaccharide was poorly formed. The detection of the erythrocytes sensitized with tuberculin-polysaccharide in the circulating blood will thus be of value for diagnosis of the activity of the tuberculous process in infancy and childhood, especially in these ages poor formation of the antibody is the rule.

How can we then evaluate the *activity* of tuberculosis in infancy and childhood when a sufficient amount of the antibody against the tuberculin-polysaccharide had been produced and all the tuberculin-polysaccharide produced from tuberculous foci was combined with the antibody, that is, when the erythrocytes sensitized with tuberculin-polysaccharide were no more demonstrated in the blood? In such a case, it is reasonable to deduce that the approximate estimation of the tuberculin-polysaccharide which was combined with the antibody in serum is of value for the evaluation of the activity of the tuberculous foci.

In the present paper we shall report a method for approximate estimation of the tuberculin-polysaccharide which was combined with the antibody in serum.

Experimental and Comment

Reagents and apparatus required

1. 0.15 *M* phosphate buffer of pH 7

2. The McIlvaine buffer of pH 3 (0.2 *M* Na_2HPO_4 4.11: 0.1 *M* citric acid 15.89)

3. 10% $Na_2HPO_4 \cdot 12H_2O$ solution

4. The diluted Immune Serum C. The Immune Serum C[2] was diluted up to 1:2000 with the 0.15 *M* phosphate buffer of pH 7.

5. The suspension of the sensitized erythrocytes. The human erythrocytes of type 0 were washed three times with the 0.15 *M* phosphate buffer of pH 7. To 0.05 cc. of the deposit of the washed erythrocytes was added 1.0 cc. of 45.6 mg% solution of tuberculin-polysaccharide[2]. The content was mixed and kept at 37°C for two hours. After the incubation, the erythrocytes sensitized with tuberculin-polysaccharide were washed three times with the 0.15 *M* phosphate buffer of pH 7. The deposit of the washed erythrocytes was suspended in 5.0 cc. of the 0.15 *M* phosphate buffer of pH 7.

6. Apparatus for the agglutination between the Immune Serum and the erythrocytes sensitized with the tuberculin-polysaccharide.

The agglutination of the erythrocytes was observed macroscopically in the hollows on a 10 mm. thick glass plate, each of which was 5 mm. in depth and 26 mm. in diameter.

Approximate estimation of the tuberculin-polysaccharide which had been combined with the antibody against same

Two sets of experiment (Sets A and B) were carried out.

Set A consisted of a series of 16 test tubes, and each of them (Nos. 1–15) contained 1.0 cc. of the diluted Immune Serum C and 1.0 cc. of the tuberculin-polysaccharide solution of different concentration, and the tube No. 16 contained distilled water instead of the tuberculin-polysaccharide solution (Cf. Table I and Note on page 300).

Set B consisted of a series of 16 test tubes (Nos. 17–32), and each of them contained 1.0 cc. of distilled water and 1.0 cc. of the tuberculin-polysaccharide solution of different concentration (Cf. Table II).

All the test tubes of Sets A and B were kept at 37°C for twenty four hours. After the incubation, the contents of each test tube were treated as follows. (For the approximate estimation of the tuberculin-polysaccharide in the serum from patients in practice, 2.0 cc. of their serum were used.)

To 2.0 cc. of the specimen to be tested—that is to say, to the contents of each of the tubes of Sets A and B—, was added 1.0 cc. of the McIlvaine buffer of pH 3. The contents, each tube without any stopper, were kept in a steam bath at 100°C for thirty minutes. After cooling them, the clear

TABLE I

Experiment of Set A

No. of test tubes	The immune serum C diluted up to 1:2000	Distilled water	Tuberculin-polysaccharide solution with different concentration (1.0 cc. per each of the tubes was used)		Agglutination between the erythrocytes sensitized with tuberculin-polysaccharide and the diluted Immune Serum C which was incubated with "the solution of released-tuberculin-polysaccharide"									
			Titer of dilution of tuberculin-polysaccharide solution with the original concentration of 456 mg%	Approximate concentration of tuberculin polysaccharide γ%	Titer of dilution of "the solution of released-tuberculin-polysaccharide"									
					1	2	4	8	16	32	64	128	256	512
1	1.0 cc.		15	30400	−	−	−	−	−	−	−	−	−	−
2	1.0 cc.		30	15200	−	−	−	−	−	−	−	−	−	−
3	1.0 cc.		60	7600	−	−	−	−	−	−	−	−	−	−
4	1.0 cc.		120	3800	−	−	−	−	−	−	−	−	−	−
5	1.0 cc.		240	1900	−	−	−	−	−	−	−	−	+	+
6	1.0 cc.		480	950	−	−	−	−	−	−	−	+	+	+
7	1.0 cc.		960	475	−	−	−	−	−	−	+	+	+	+
8	1.0 cc.		1920	$475\times\frac{1}{2}$	−	−	−	−	+	+	+	+	+	+
9	1.0 cc.		3840	$475\times\frac{1}{4}$	−	+	+	+	+	+	+	+	+	+
10	1.0 cc.		7680	$475\times\frac{1}{8}$	+	+	+	+	+	+	+	+	+	+
11	1.0 cc.		15360	$475\times\frac{1}{16}$	+	+	+	+	+	+	+	+	+	+
12	1.0 cc.		30720	$475\times\frac{1}{32}$	+	+	+	+	+	+	+	+	+	+
13	1.0 cc.		61440	$475\times\frac{1}{64}$	+	+	+	+	+	+	+	+	+	+
14	1.0 cc.		122880	$475\times\frac{1}{128}$	+	+	+	+	+	+	+	+	+	+
15	1.0 cc.		245760	$475\times\frac{1}{256}$	+	+	+	+	+	+	+	+	+	+
16	1.0 cc.	1.0 cc.			+	+	+	+	+	+	+	+	+	+

TABLE II

Experiment of Set B

No. of test tubes	Distilled water	Tuberculin-polysaccharide solution with different concentration (1.0 cc. per each tube was used)		Agglutination between the erythrocytes sensitized with tuberculin-polysaccharide and the diluted Immune Serum C which was incubated with "the solution of released-tuberculin-polysaccharide"									
		Titer of dilution of tuberculin-polysaccharide solution with the original concentration of 456 mg%	Approximate concentration of tuberculin-polysaccharide γ%	Titer of dilution of "the solution of released-tuberculin-polysaccharide"									
				1	2	4	8	16	32	64	128	256	512
17		15	30400	−	−	−	−	−	−	−	−	−	−
18		30	15200	−	−	−	−	−	−	−	−	−	−
19		60	7600	−	−	−	−	−	−	−	−	−	−
20		120	3800	−	−	−	−	−	−	−	−	−	−
21		240	1900	−	−	−	−	−	−	−	−	−	+
22		480	950	−	−	−	−	−	−	−	−	+	+
23		960	475	−	−	−	−	−	−	−	+	+	+
24		1920	$475\times\frac{1}{2}$	−	−	−	−	−	−	+	+	+	+
25		3840	$475\times\frac{1}{4}$	−	−	−	−	−	+	+	+	+	+
26		7680	$475\times\frac{1}{8}$	−	−	−	−	+	+	+	+	+	+
27		15360	$475\times\frac{1}{16}$	−	−	−	+	+	+	+	+	+	+
28		30720	$475\times\frac{1}{32}$	−	−	+	+	+	+	+	+	+	+
29		61440	$475\times\frac{1}{64}$	−	+	+	+	+	+	+	+	+	+
30		122880	$475\times\frac{1}{128}$	+	+	+	+	+	+	+	+	+	+
31		245760	$475\times\frac{1}{156}$	+	+	+	+	+	+	+	+	+	+
32	2.0 cc.			+	+	+	+	+	+	+	+	+	+

filtrate was obtained from each of the tubes, and adjusted to pH 7 by the addition of 10% Na_2HPO_4 solution. The final volume of the filtrate was made up to 1.0 cc. with distilled water—this was " the solution of released-tuberculin-polysaccharide "—. Each of " the solution of released-tuberculin-polysaccharide " was diluted with the 0.15 *M* phosphate buffer of pH 7 to get a series of the dilution of 1, 2, 4, 8, 16, 32, 64, 128, 256 and 512 times. Six drops of " the diluted solution of released-tuberculin-polysaccharide " were put into each of the hollows on a glass plate, and to each of them were added two drops of the diluted Immune Serum C. The contents were kept at 37°C for three hours. After the incubation, to each of the hollows was added one drop of the suspension of the erythrocytes sensitized with tuberculin-polysaccharide solution, then the incubation at 37°C for two hours was carried out again. The agglutination was observed macroscopically.

The results of the experiment of Sets A and B were given in Tables I, II and III.

In the experiment of Set B, in which the tuberculin-polysaccharide solution (without the combination with the antibody prior to the estimation) was estimated, the inhibition of agglutination of the sensitized erythrocytes was observed in the cases of Nos. 17–29, but not in those of Nos. 30–32 (Cf. Table II). These findings showed that neutralization of the antibody in the diluted Immune Serum C was able to be achieved by the addition of the tuberculin-polysaccharide with the concentration of ca. 7 γ% or more. In other words, in the case of the estimation of tuberculin-polysaccharide which had been dissolved in the phosphate buffer, the concentration of the tuberculin-polysaccharide would be found from the pattern of the agglutination of the sensitized erythrocytes (Cf. Table II). For instance, such a pattern of the agglutination as $-+++++++++$ and $+$ stood for ca. 7 γ% in the concentration of the tuberculin-polysaccharide in the specimen tested ; $--++++++++$ and $+$, for ca. $7\times2\,\gamma$% ; $---+++++++$ and $+$, for ca. $7\times2\times2\,\gamma$%, and and so on (Cf. Table II).

On the other hand, in the experiment of Set A, in which the tuberculin-polysaccharide had been combined with the antibody in serum prior to the estimation, such a pattern of the agglutination as $-++++++++$ and $+$ was observed in the case of No. 9, in which the theoretical concentration of the tuberculin-polysaccharide was of approximately $7\times16\,\gamma$% (Cf. Table I). In other words, when the tuberculin-polysaccharide had been combined with the antibody in serum prior to the estimation, the recovery of the tuberculin-polysaccharide was found to be 1/16, 1/4 and 1/2 of the theoretical concentrations of 118, 237, and 950–1900 γ% respectively. Until a method for giving more staisfactory recovery rate of the tuberculin-

TABLE III

Relation between the Pattern of the Agglutination and the Concentration of Tuberculin-Polysaccharide in Serum

Pattern of agglutination	Approximate concentration of tuberculin-polysaccharide in serum (γ%)
+ + + + + + + + + +	0 or below 118
− + + + + + + + + +	$475\times\frac{1}{4}\fallingdotseq 118$*
− − + + + + + + + +	$118+\frac{118}{3}\fallingdotseq 157$*
− − − + + + + + + +	$118+\frac{2\times 118}{3}\fallingdotseq 196$*
− − − − + + + + + +	$475\times\frac{1}{2}\fallingdotseq 237$
− − − − − + + + + +	$237+\frac{237}{2}\fallingdotseq 355$*
− − − − − − + + + +	475
− − − − − − − + + +	950
− − − − − − − − + +	1900
− − − − − − − − − −	3800 or more

* Calculated from the nearest two figures.

polysaccharide which had been combined with the antibody in serum will have been devised, we shall be, as we were, obliged to content ourselves by assuming tentatively the results given in Table III, which, basing upon the findings in Table I, showed a relation between the pattern of the agglutination and the concentration of the tuberculin-polysaccharide in the serum tested (Cf. Table III). The tuberculin-polysaccharide in the serum from tuberculous patients was thus approximately estimated.

Note: In the experiment of Set A, it was confirmed experimentally that, when the Immune Serum C was incubated with the tuberculin-polysaccharide solution of 45.6 mg%, sufficient amount of the antibody against the tuberculin-polysaccharide remained uncombined in the Immune Serum C.

CONCLUSIONS

A method for the approximate estimation of the tuberculin-polysaccharide, which had been combined with the antibody against the tuberculin-polysaccharide in the serum, was reported.

By using this method, "activity diagnosis" of tuberculosis in infancy and childhood will be possible, even in the case in which the antibody is developing in serum, in other words when there is no more identification of the sensitized erythrocytes in blood.

References

1) Arakawa, Oota & Sato, Tohoku J. Exp. Med., 1958, **68**, 99.
2) Arakawa, Oota & Sato, *ibid.*, 1958, **68**, 93, 105.